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14. ABSTRACT

Progression to castrate resistant prostate cancer for men with advanced prostate cancer (PC) results after the initiation of androgen deprivation therapy (ADT). One underutilized therapeutic strategy that has the potential to dramatically improve outcomes is eradicating the persistent cancer cells that remain after ADT and likely play a key role in the development of castration-resistant PC. The initiation of ADT induces susceptibilities in PC cells that make them amenable to synergistic treatment and improved cell killing.

15. SUBJECT TERMS
prostate cancer, androgen deprivation therapy, senescence, proteotoxic stress, xenograft models, metformin, synthetic lethality

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Table of Contents

Page

1. **Introduction…… 4**
2. **Keywords……. 5**
3. **Accomplishments……...……... 5**
4. **Impact……...…… 10**
5. **Changes/Problems...…..…… 10**
6. **Products……..……..…..……. 10**
7. **Participants & Other Collaborating Organizations…… 11**
8. **Special Reporting Requirements…… 13**
9. **Appendices…… 14**

Title: **Synthetic lethal metabolic targeting of senescent cells after androgen deprivation therapy**

1. Introduction

Progression to castrate resistant prostate cancer for men with advanced prostate cancer (PC) results after the initiation of androgen deprivation therapy (ADT). One underutilized therapeutic strategy that has the potential to dramatically improve outcomes is eradicating the persistent cancer cells that remain after ADT and likely play a key role in the development of castration-resistant PC. The initiation of ADT induces susceptibilities in PC cells that make them amenable to synergistic treatment and improved cell killing.

Androgen withdrawal in murine xenografts and human PC tissues is associated with a decrease in the proliferative index, but surprisingly low levels of apoptosis. We and others have demonstrated that a substantial portion of these persistent cells express markers of cellular senescence, a terminal growth arrest characterized by exit from the cell cycle and senescence-associated β-galactosidase expression. These senescent cells, although not proliferating, generate a protumor response, the senescent secretory phenotype, that may be detrimental to the patient and must be removed. However, the unique metabolic phenotype expressed by these persistent senescent cells is characterized by increased protein synthesis and notably an amplified proteotoxic stress response (PSR), a conserved survival pathway characterized by induction of multiple heat shock protein (Hsp) families coordinated by the master transcriptional regulator Hsf1. It is our overall hypothesis that the new senescent phenotype induced in prostate cancer cells by ADT may result in unique vulnerabilities to drugs targeting pathways such as the PSR that are critical for survival in the senescent state.

In preliminary data activation of the PSR in these residual cancer cells may represent a pathway critical for the survival of senescent PC cells. Further experiments have identified one agent, metformin, a widely-used, nontoxic oral antidiabetic drug that we propose to repurpose as synthetic lethal therapy in combination with ADT. We postulate that that metformin is synthetic lethal with ADT because it disables the principal PSR pathway mediated by Hsf1 in senescent PC cells that are already experience high levels of proteotoxic stress.

In Aim 1 we will examine the activity of metformin in eradicating senescent PCs following ADT in cellular models. In addition, we will determine whether metformin's actions are specifically mediated by inactivation of Hsf1 and resultant disruption of the PSR cell survival pathway mediated by Hsp27, Hsp70 and Hsp 90. In vitro and xenograft PC models utilizing overexpression of a phosphorylation-resistant Hsf1 mutant will be used to interrogate the specific role of the HSf1-mediated PSR in the synthetic lethal response. PC has a variable response to ADT. The ability of metformin to clear senescent cancer cells after ADT will be examined in Aim 2 in a series of human PC xenografts that exhibit variable responses to ADT. We will utilize a xenograft system consisting of human prostate cancer tumors that can be exposed to drug combinations in a physiologically relevant setting, the growth easily tracked, and the tumor readily harvested for detailed examination. Experiments will test whether synchronous ADT-metformin or their stepwise use leads to better tumor regression and longer survival. In addition, markers of response will be investigated in the tumors focusing on the Hsps examined in Aim 1. Finally, in Aim 3 we will employ a health sciences research approach using the National Department of Veterans Affairs Corporate Data Warehouse to investigate a retrospective cohort of patients on ADT (~260,000 men), 8% of whom are on metformin (~21,000), to determine

PC-specific mortality, biochemical recurrence-free survival and skeletal related events. This will provide further evidence for the implementation of this novel synthetic lethal therapeutic strategy.

Our studies have the potential to lead to a new treatment paradigm for PC by specifically targeting a unique vulnerability of senescent PCs (the PSR) that persist following ADT and likely contribute to androgen-resistance. The proposed study directly addresses mechanisms of resistance for men with high-risk cancer and furthermore, since metformin may mitigate the metabolic side effects of ADT, may improve the physical health of men with PC. When completed our new synthetic lethal approach to PC can be readily translated into the clinic since both ADT and metformin are safe and currently in use.

2. Keywords

prostate cancer, androgen deprivation therapy, senescence, proteotoxic stress, xenograft models, metformin, synthetic lethality

3. Accomplishments

SPECIFIC AIM 1:

Major Task 1: Determine whether the ADT-metformin synergistic response is mediated via disruption of the Hsf1-mediated proteolytic stress response (PSR).

Subtask 1: Characterize the effects of metformin on the viability of senescent PC cells following ADT (Jarrard/Cryns)

- PC cells will be treated with vehicle or biculatamide for 4 days followed by metformin or vehicle for 2-4 days
- Score senescent PC cells using SA-β-gal activity, GLB1 immunostaining and flow cytometry
- Evaluate apoptosis using co-immunofluorescence with GLB1 and active caspase-3 Ab and by annexin V labeling of GLB1-flourescent PC cells
- Cell lines: LNCaP, CWR22Rv1, VCaP in subtasks 1-3

Ongoing. We have optimized dosing and time to achieve a maximal coefficient index for synthetic lethality for 3 cell lines, LNCaP, CWR22Rv1 and LAPC4. VCaP was not utilized because of variable androgen responsiveness. LAPC4 was used as an alternate.

Senescence has been quantitated using SA-β-gal activity, and flow cytometry as well as western for p16 and p27. Apoptosis occurs maximally at 48 hr after exposure to metformin as noted in the cell line LAPC4 Figure 1.

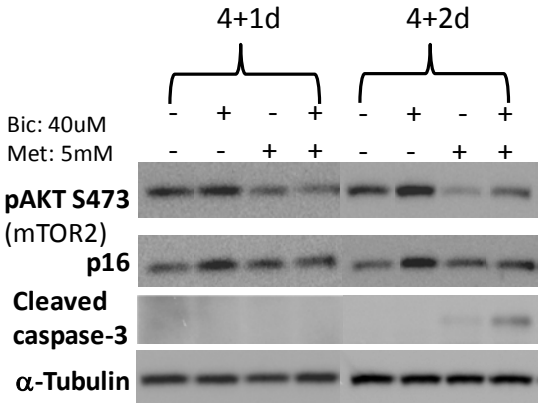

Figure 1 legend: LAPC4 Cells were treated with bicalutamide for 4 days, and then bicalutamide was removed followed by treatment of metformin for 1 or 2 days. Induction of p16 is a senescent marker. CC3 indicates increased apoptosis.

Senescence occurs in a subpopulation and we are currently investigating options for selecting cells. Sorting for SA-B-gal expression is too laborious and not optimal. We also attempted to flow sort based on size but this approach is too slow. We are currently using senescence surface marker to isolate senescent cells. The expression of plasma membrane-associate proteins VSP26A, DCR2 and B2M increased in senescent cells, we are using biotinylated antibodies against these proteins, capturing with streptavidin magnetic beads, and isolating with a microchamber.

Subtask 2: Characterize the effects of metformin on the PSR of senescent PC cells following ADT (Cryns/Jarrard).

- PC cells will be treated with ADT and/or metformin as in subtask 1
- Collect GLB1-fluorescent and non-fluorescent cells by FACs and determine Hsf1, Hsp27, Hsp70 and Hsp90 mRNA and protein levels.
- Perform co-immunofluorescence with GLB1 and Hsf1, Hsp27, Hsp70, Hsp90, p-AMPK and AMPK
- Determine whether AMPK binds directly to Hsf1 by co-immunoprecipitation.

Milestones: We predict that metformin will increase binding of AMPK to Hsf1 and inhibit the PSR.

Ongoing. We have examined the proteotoxic stress response in multiple prostate cancer cell lines in response to androgen deprivation therapy with and without metformin treatment. Analyses of the entire population of treated prostate cancer cells has yielded variable results depending on prostate cancer cell line and individual heat shock proteins with regard to the effects of both androgen deprivation and metformin treatment. However, these studies did not specifically examine the effects of these interventions on the subpopulation of senescent prostate cancer cells due to technical difficulties noted in Subtask 1. Using the modified approach outlined in Subtask 1, we will isolate the senescent subpopulation after treatment, and we expect to complete these studies in year 2 of the project. Figure 2.

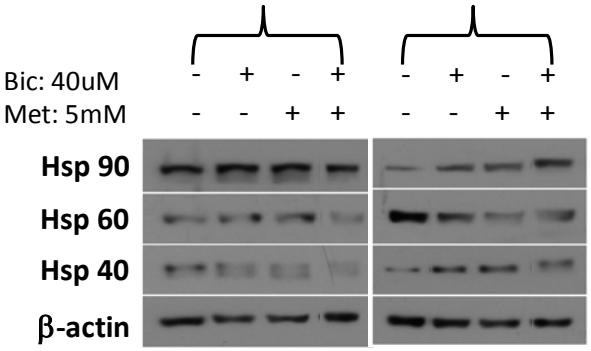

Figure 2 legend: LAPC4 Cells were treated with bicalutamide for 4 days, and then bicalutamide was removed followed by treatment of metformin for 1 or 2 days.

Subtask 3: Determine the functional role of site-specific phosphorylation of Hsf1 on the effects of metformin on senescent PCs following ADT (Cryns/Jarrard).

- Stably transduce PC cells with vector, WT Hsf1 or mutant S121A Hsf1 by lentiviral transduction.

- Treat PC cells stably expressing vector, WT or S121A mutant Hsf1 with ADT and/or metformin as in subtask 1
- Perform cell viability (subtask 1) and molecular characterization (subtask 2) assays on senescent PCs and the entire population of PCs.

Milestones: We predict that the S121A mutant Hsf1 will abrogate the effects of metformin on cell death and PSR following ADT.

Pending.

Subtask 4: Determine the function of site-specific phosphorylation of Hsf1 on the antitumor effects of ADT and metformin in vivo (Jarrard/Cryns).

- Male nude mice with LnCaP and CWR22rv1 flank tumors stably expressing WT or S121A mutant Hsf1 will be randomized to 4 groups (8 mice per group): (1) vehicle + sham operation; (2) vehicle + castration; (3) metformin + sham operation; and (4) metformin + castration. Metformin will be tried simultaneously (groups 3 and 4) or sequentially (groups 5 and 6). Tumor size will be assessed weekly and serum PSA recorded. To assess senescent cell clearing, a parallel experiment will be performed using the same 4 treatment groups (8 mice per group) except that mice will be euthanized 4 weeks after castration and tumors harvested for analysis. (16X(6X3)) for 2 experiments (total 576)
- Mouse tissues will be analyzed for SA-β-gal activity, HP1γ, GLB1, p27, Hsp27, Hsp70, Hsp90, Ki67, active caspase-3 and TUNEL staining

Pending.

Major Task 2: Examine the synthetic lethal response involving ADT-metformin in vivo in cancers of variable androgen sensitivity and test markers of response.

Subtask 1: To determine the optimal schedule for combining ADT and metformin and assess whether metformin eradicates senescent PC cells following ADT (Jarrard). We will specifically determine whether sequential (i.e. ADT then metformin) is superior to simultaneous (ADT and metformin) and whether senescent cells are being cleared from the tumors with the addition of metformin.

- Obtain approval by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC). (Months 1-4)
- Four groups will be randomized using 3 xenografts (58, 23.1 and 81) when flank tumors are 100mm3 to examine the i) Castrated mice (16 animals x 3 xenograft lines), ii) Mice placed on Metformin (mg/kg) given orally daily in intact sham operated mice (16 X 3 xenograft lines), iii) Mice (16 x 3 xenograft lines) will be castrated and placed on Metformin (mg/kg) given orally daily 10d post-castration and iv) Mice (16 x 3 xenograft lines) will be castrated and placed on Metformin (50mg/kg-1) given orally daily immediately post-castration. Post-ADT tumor size will be assessed weekly and serum PSA levels (Total 16X(4X2)) for 2 experiments (total 256). Grand total 832 plus 10% to counter attrition and loss of animals (915).

- Half of the animals (8) in each group will be harvested 4wk after castration to permit an assessment of senescence cell clearing after metformin. The remaining animals will continue to be monitored for survival
- Senescent cell clearing will be measured as described above in 1D using primarily Vectra quantitation of immunohistochemistry. Apoptosis will be examined as described above.

Ongoing studies on track. Animal approval has been obtained and the studies are ongoing. The xenograft lines had to be obtained from Univ Washington and expanded. Test dosing of metformin performed successfully. Currently tumors implanted in experimental mice and tumors growing.

Subtask 2: To determine whether PSR markers predict improved response to ADT-Metformin (Jarrard/Cryns). Xenograft tumors from the 2A will be sectioned and immunofluorescence will be used and quantitated using the automated Vectra™ system for Hsp27, Hsp70 and Hsp90. The proteolytic stress response(PSA) represented by these 3 genes in castrated animals harvested at 4wk (group i) will be statistically compared to tumor response, survival, PSA, and other markers including GLB1 in ADT-Metformin groups (iii and iv).

Pending.

Major Task 3: Determine whether metformin combined with ADT results in improved cancer-specific survival and longer time to secondary interventions in patients on these agents.

Subtask 1: We propose to utilize a robust observational cohort from the national Veterans Affairs (VA) database to specifically evaluate our hypothesis that metformin improves PC response to ADT, thereby directly examining the patient relevance of our preclinical data in validated patient population. Approvals (Jarrard/Richards).

Completed.

Subtask 2: Data collection, organization with exclusion and inclusion from 2000-2008 (Jarrard/Richards).

Completed.

Subtask 3: Analysis of primary and secondary predictive variables (Jarrard/Richards). Evaluate and control for other covariates including other diabetes medication administration history, age, race, Charlson-comorbidity score, agent orange exposure, family history of prostate cancer, tobacco use, blood type, local therapy (surgery or radiation), date of prostate cancer diagnosis, stage at diagnosis, Gleason score, and other medication administration history (finasteride, aspirin, and docetaxol).

Completed.

Milestone(s) Achieved:

Using national Veterans Affairs databases, we identified all men diagnosed with PCa between 2000-2008 that were treated with ADT with follow-up through October of 2015. We excluded patients that were treated with ADT for $\leq$6 months or were receiving ADT concurrently with localized radiation therapy. We split these patients into three cohorts: 1. Patients without diabetes 2. Diabetics on metformin 3.

Diabetics not treated with metformin. Our primary outcome was overall survival (OS) and secondary outcomes included skeletal related events (SRE) and prostate-cancer specific survival. Cox proportional hazards ratios were calculated for overall and disease specific survival.

The total cohort after exclusions consisted of 87,344 patients of which 53,893 (61%) were non-diabetics, 14,517 (17%) were diabetics on metformin, and 18,934 (22%) were diabetics not receiving metformin. The mean age was 75 $\pm$11 years in the non-diabetics, 71 $\pm$12 in the diabetics on metformin, and 75 $\pm$10 in the diabetics no metformin ($p<0.001$). The median OS was 7.1 years in the non-diabetics, 9.1 years in the diabetics on metformin, and 7.4 years in the diabetics not treated with metformin.

Multivariable Cox proportional hazards analysis assessing for predictors of overall survival showed improved survival in diabetics on metformin (HR 0.77, 95% CI 0.74-0.81) vs. diabetics not treated with metformin (HR 0.99, 95% CI 0.95-1.03) with non-diabetics as referent group. Multivariable Cox proportional hazards analysis assessing for predictors of SRE revealed no association between metformin use (HR 0.99, 95% CI 0.92-1.07) and SRE. Lastly, multivariable Cox proportional hazards analysis assessing for predictors of prostate-cancer specific survival showed improved survival in diabetics on metformin (HR 0.72, 95% CI 0.67-0.78) and to a lesser effect diabetics not treated with metformin (HR 0.87, 95% CI 0.81- 0.93) with non-diabetics as referent group.

We conclude that metformin use in Veterans with advanced prostate cancer receiving ADT is associated with improved OS and cancer-specific survival. The impact of metformin in prostate cancer patients should be evaluated in a prospective clinical trial.

Completed and abstract presented at the American Urological Association Meeting May 2017 and the GU ASCO meeting Feb 2017. Paper currently in preparation.

Opportunities for training and professional development?

These include a post-doctoral fellow Dr Shiva Damoradan currently in the laboratory. Additional trainees include Nathan Damaschke a graduate student who performed

How were the results disseminated to communities of interest?

Abstract presentation at the American Urological Association meeting 5/2017 Boston MA.

• What do you plan to do during the next reporting period to accomplish the goals and objectives?

We plan to adhere to the proposed SOW with the exceptions noted under accomplishments.

4. Impact

Androgen deprivation therapy (ADT) induced by surgical or chemical castration limits the growth of androgen-responsive tissues, but is not curative and ultimately castration-resistant PC results. An innovative therapeutic niche that has not been successfully exploited to date is the persistent population of PC cells after ADT treatment that likely contributes to castration-resistant PC. We and others have demonstrated that a substantial portion of these persistent cells express markers of cellular senescence, a terminal growth arrest characterized by exit from the cell cycle. Persistent senescent cells exhibit a senescent-associated secretory phenotype, which results in enhanced protein translation and the accumulation of misfolded proteins, thereby activating a conserved proteotoxic stress response (PSR) characterized by induction of multiple heat shock protein (Hsp) families coordinated by the master transcriptional regulator Hsf1 that enables cell survival. We propose that this intrinsic PSR may represent a potential 'Achilles heel' that may be exploited to drive persistent PC cells into apoptosis using a synthetic lethal approach. Intriguingly, the safe and widely used diabetes oral agent metformin was recently reported to inhibit the Hsf1-mediated PSR. **The induction of cell senescence using ADT, followed by a synthetic lethal therapy approach using metformin to drive these senescent cells into apoptosis by disrupting the PSR, is a transformative concept has not been addressed to date**. Although components of the PSR such as Hsp27 have been targeted previously, we postulate that our approach will be more effective because it targets the global PSR network, not just one of its many downstream mediators. Notably, metformin is an inexpensive drug with documented safety, even in nondiabetic patients that ***we propose to repurpose*** as synthetic lethal cancer therapy that can be readily translated into the clinic. This proposal addresses the PCRP overarching challenge of mechanisms of resistance in men with high-risk PC.

5. Changes Problems

We are using a modified approach to identify senescent prostate cancer cells after treatment as noted under Major Task 1, Subtask 1.

6. Products

Abstracts:

Metformin Use Associated with Improved Survival in Veterans with Advanced Prostate Cancer: A Large Observational Study. Kyle A. Richards, Jinn-ing Liou, Vincent Cryns, Tracy M. Downs, E. Jason Abel, and David F. Jarrard (American Urological Association Meeting. Boston MA 2017)

Publications:

Strekalova E, Malin D, Rajanala H, **Cryns VL**. Metformin sensitizes triple-negative breast cancer to proapoptotic TRAIL receptor agonists by suppressing XIAP expression. *Breast Cancer Res Treat* 2017;163:435-447.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Senior key personnel have been working on the project since the initiation of the project with no changes.

The following individuals have worked on the project:

Name: David F. Jarrard, MD

Project Role: Principal Investigator

Researcher Identifier (e.g., ORCID ID): 0000-0001-8444-7165

Nearest person month worked: 1.2

Contribution to Project: David Jarrard has conceived and designed the study, reviewed all of the data and the analysis of all of the results on the project, wrote and revised the manuscript.

Name: Vince Cryns, MD

Project Role: Co-Principal Investigator

Researcher Identifier (e.g., ORCID ID): 0000-0003-0355-2268

Nearest person month worked: 2

Contribution to Project: Vince Cryns has conceived and designed the study, reviewed all of the data and the analysis of all of the results on the project, wrote and revised the manuscript.

Name: Kyle Richards, MD

Project Role: Co- Investigator

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 0.3

Contribution to Project: Dr Richards has reviewed the data and the analysis of all of Aim 3 on the project, co-wrote and revised the manuscript.

Name: Shiva DAMODARAN

Project Role: Post-Doctoral Fellow

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 4

Contribution to Project: Shiva has optimized the animal xenografts and metformin treatment and is currently performing the animal studies.

Name: Bing Yang, MD, PhD

Project Role: Researcher

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 3.6

Contribution to Project: Bing Yang has prepared all the PCa cell lines used in this study and performed the analysis on the cell lines, organized the data and assisting with the mouse studies.

Name: Joe Gawdzik, PhD

Project Role: Post-Doctoral Fellow

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 1

Contribution to Project: Joe has assisted with the cell culture experiments for Task 1 and data analysis and collection.

Name: Jinn-ing Liou

Project Role: Researcher

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 0.3

Contribution to Project: Jinn-ing has generated the data and the analysis of all of Aim 3 on the project, co-wrote and revised the manuscript.

Name: Dmitry Malin, PhD

Project Role: Associate Scientist

Researcher Identifier (e.g., ORCID ID): 0000-0002-5728-7511

Nearest person month worked: 6

Contribution to Project: Dmitry Malin has analyzed human PCa cell lines for markers of proteotoxic stress in response to androgen deprivation therapy with or without metformin treatment and assisted with the design of these experiments.

8. SPECIAL REPORTING REQUIREMENTS:

Duplicative reports have been submitted for both the Initiating PI ad the Partnering PI

9. APPENDICES:

Abstract and manuscript.

Metformin Use Associated with Improved Survival in Veterans with Advanced Prostate Cancer: A Large Observational Study

Kyle A. Richards, Jinn-ing Liou, Vincent Cryns, Tracy M. Downs, E. Jason Abel, and David F. Jarrard

Size: The size of the abstract is limited to 2,280 characters not including spaces.

This includes title, body of abstract, tables and graphics. Tables are calculated at 225 characters per table. Graphics are calculated at 225 characters per graphic.

Background: Metformin is a commonly prescribed 1st line glucose-lowering therapy for patients with type 2 diabetes mellitus. There is increasing clinical data supporting the anti-neoplastic effects of metformin for various cancers including prostate cancer (PCa). In addition, we hypothesize that metformin plus androgen deprivation therapy (ADT) may be synergistic in men with advanced PCa. Hence the objective of this study was to assess the impact of metformin plus ADT in a retrospective cohort of Veterans with advanced PCa.

Methods: Using national Veterans Affairs databases, we identified all men diagnosed with PCa between 2000-2008 that were treated with ADT with follow-up through October of 2015. We excluded patients that were treated with ADT for ≤6 months or were receiving ADT concurrently with localized radiation therapy. We split these patients into three cohorts: 1. Patients without diabetes 2. Diabetics on metformin 3. Diabetics no metformin. Our primary outcome was overall survival (OS) and secondary outcomes included skeletal related events (SRE) and prostate-cancer specific survival. Cox proportional hazards ratios were calculated for overall and disease specific survival.

Results: The total cohort after exclusions consisted of 87,344 patients of which 53,893 (61%) were non-diabetics, 14,517 (17%) were diabetics on metformin, and 18,934 (22%) were diabetics not receiving metformin. The mean age was 75 ±11 years in the non-diabetics, 71 ±12 in the diabetics on metformin, and 75 ±10 in the diabetics no metformin ($p<0.001$). The median OS was 7.1 years in the non-diabetics, 9.1 years in the diabetics on metformin, and 7.4 years in the diabetics no metformin.

Multivariable Cox proportional hazards analysis assessing for predictors of overall survival showed improved survival in diabetics on metformin (HR 0.77, 95% CI 0.74-0.81) vs. diabetics no metformin (HR 0.99, 95% CI 0.95-1.03) with non-diabetics as referent group. Multivariable Cox proportional hazards analysis assessing for predictors of SRE revealed no association between metformin use (HR 0.99, 95% CI 0.92-1.07) and SRE. Lastly, multivariable Cox proportional hazards analysis assessing for predictors of prostate-cancer specific survival showed improved survival in diabetics on metformin (HR 0.72, 95% CI 0.67-0.78) and to a lesser effect diabetics no metformin (HR 0.87, 95% CI 0.81- 0.93) with non-diabetics as referent group.

Conclusion: Metformin use in Veterans with advanced prostate cancer receiving ADT is associated with improved OS and cancer-specific survival. The impact of metformin in prostate cancer patients should be evaluated in a prospective clinical trial.

Breast Cancer Res Treat (2017) 163:435–447
DOI 10.1007/s10549-017-4201-0

PRECLINICAL STUDY

Metformin sensitizes triple-negative breast cancer to proapoptotic TRAIL receptor agonists by suppressing XIAP expression

Elena Strekalova[1] · Dmitry Malin[1] · Harisha Rajanala[1] · Vincent L. Cryns[1]

Received: 18 January 2017 / Accepted: 13 March 2017 / Published online: 21 March 2017

Abstract
Purpose Despite robust antitumor activity in diverse preclinical models, TNF-related apoptosis-inducing ligand (TRAIL) receptor agonists have not demonstrated efficacy in clinical trials, underscoring the need to identify agents that enhance their activity. We postulated that the metabolic stress induced by the diabetes drug metformin would sensitize breast cancer cells to TRAIL receptor agonists.
Methods Human triple (estrogen receptor, progesterone receptor, and HER2)-negative breast cancer (TNBC) cell lines were treated with TRAIL receptor agonists (monoclonal antibodies or TRAIL peptide), metformin, or the combination. The effects on cell survival, caspase activation, and expression of TRAIL receptors and the antiapoptotic protein XIAP were determined. In addition, XIAP was silenced by RNAi in TNBC cells and the effects on sensitivity to TRAIL were determined. The antitumor effects of metformin, TRAIL, or the combination were evaluated in an orthotopic model of metastatic TNBC.
Results Metformin sensitized diverse TNBC cells to TRAIL receptor agonists. Metformin selectively enhanced the sensitivity of transformed breast epithelial cells to TRAIL receptor agonist-induced caspase activation and apoptosis with little effect on untransformed breast epithelial cells. These effects of metformin were accompanied by robust reductions in the protein levels of XIAP, a negative regulator of TRAIL-induced apoptosis. Silencing XIAP in TNBC cells mimicked the TRAIL-sensitizing effects of metformin. Metformin also enhanced the antitumor effects of TRAIL in a metastatic murine TNBC model.
Conclusions Our findings indicate that metformin enhances the activity of TRAIL receptor agonists, thereby supporting the rationale for additional translational studies combining these agents.

Keywords Metformin · Breast cancer · TRAIL · Metastasis · Apoptosis · Therapeutics

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) and agonistic mAbs targeting its proapoptotic receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) selectively activate the caspase-8/10-mediated extrinsic apoptotic pathway in transformed cells and exhibit robust antitumor effects in diverse murine models of cancer [1, 2]. Despite promising preclinical results, TRAIL receptor agonists have failed to demonstrate significant efficacy, either alone or in combination with chemotherapy, in multiple clinical trials in advanced malignancies [3–8]. These disappointing results in clinical trials have been attributed to a number of factors, including de novo and/or acquired resistance to TRAIL receptor agonists, inadequate receptor oligomerization on ligand binding, limiting procaspase-8/10 activation, and lack of biomarkers to predict treatment response [1, 2].

Electronic supplementary material The online version of this article (doi:10.1007/s10549-017-4201-0) contains supplementary material, which is available to authorized users.

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In an effort to enhance the activity of TRAIL receptor agonists, these proapoptotic agents have been combined with TRAIL-sensitizing agents (e.g., histone deacetylase inhibitors, PPARγ agonists, aspirin, mTOR, and other kinase inhibitors) to augment its antitumor effects [9–13]. More recently, we have identified a novel nutritional

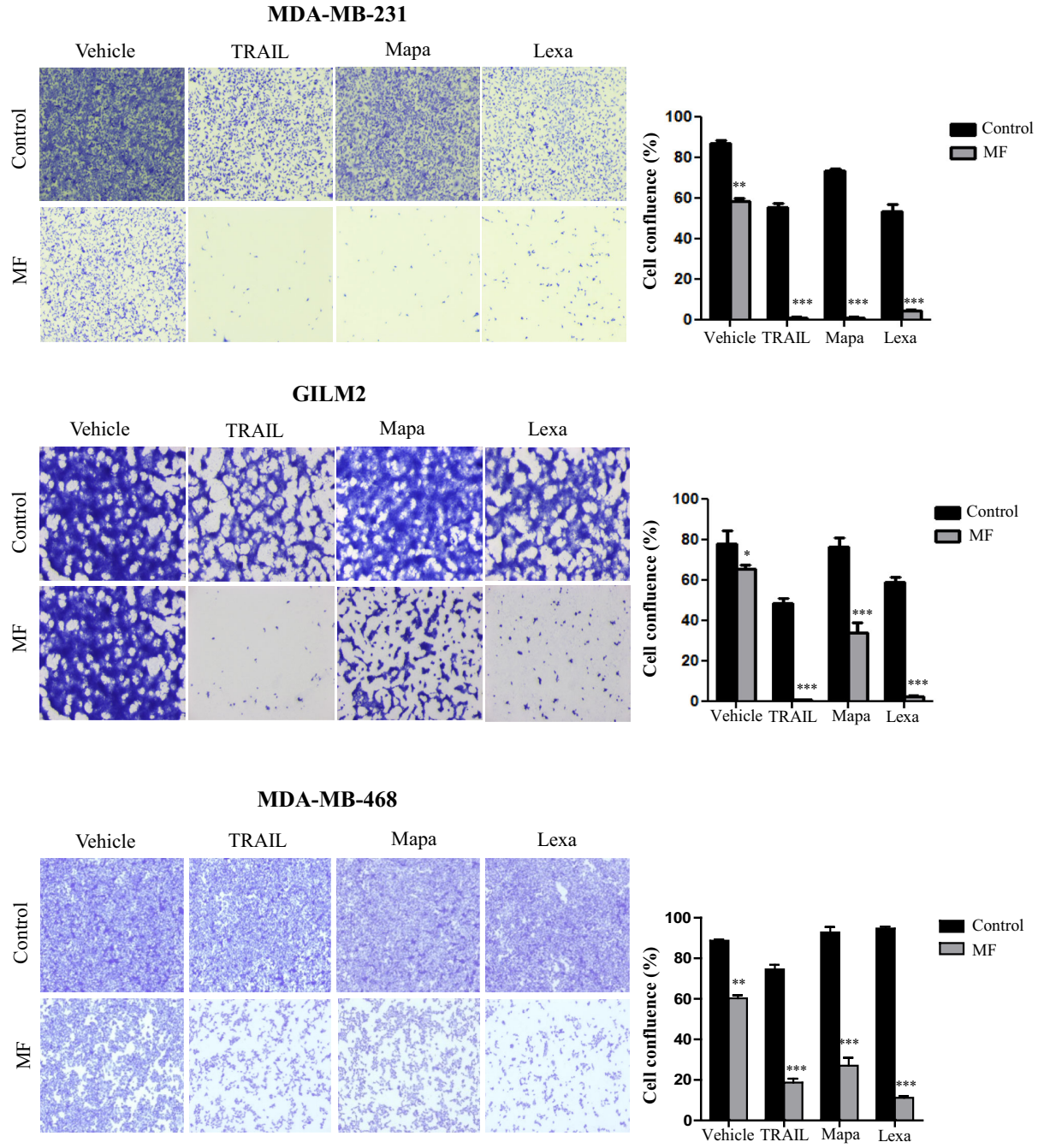


Fig. 1 Metformin sensitizes TNBC cells to TRAIL receptor agonists. Crystal violet cell survival assay of TNBC cells preincubated with vehicle or metformin (1 mM for MDA-MB-468 cells or 5 mM for MDA-MB-231-mCherry and GILM2 cells) for 48 h, and treated with vehicle or TRAIL, mapatumumab, or lexatumumab (each 1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for an additional 24 h (MDA-MB-231-mCherry and GILM2 cells) or 48 h (MDA-MB-468 cells). *Left panels* representative images. *Right panels* the percentage confluence of crystal violet-positive cells was scored (mean ± SEM, $n = 3$). In all panels, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

intervention that selectively sensitizes breast cancer cells to TRAIL-R2 agonists [14]. Specifically, we demonstrated that depletion of the essential amino acid methionine metabolically primes breast cancer cells to respond to the agonistic TRAIL-R2 mAb lexatumumab by increasing cell surface expression of TRAIL-R2. Moreover, dietary methionine restriction enhanced the antitumor activity of lexatumumab against mammary tumors and lung metastases in an orthotopic model of metastatic breast cancer [14]. Hence, methionine restriction metabolically primes breast cancer cells to targeted agents that activate cell death by exposing a targetable vulnerability, namely enhanced cell surface expression of TRAIL-R2.

Intriguingly, the diabetes medication metformin mimics many of the effects of methionine restriction, including disruption of methionine metabolism via inhibition of the functionally linked folate cycle in the one-carbon metabolic pathway, inhibition of the mechanistic target of rapamycin (mTOR), broad antitumor activity, improved insulin sensitivity, and prolonged lifespan [15–19]. Metformin use has been associated with reduced incidence of a broad range of tumor types and reduced cancer mortality in many epidemiologic studies [19–22]. However, the antitumor mechanisms of metformin are not well understood. Both direct tumor effects (activation of AMPK with resultant inactivation of mTORC1, inhibition of mitochondrial complex I, and suppression of nuclear translocation of NFκB and Stat3 phosphorylation) and indirect systemic effects (reduction in insulin and IGF-1 levels) have been reported and implicated in its antitumor effects [18, 19]. Consistent with preclinical findings, metformin treatment of newly diagnosed breast cancer patients prior to surgery improves metabolic indices, increases tumor apoptosis, and decreases tumor proliferation [23].

Given the similarities noted between methionine restriction and metformin, we postulated that metformin may also metabolically prime breast cancer cells to respond to proapoptotic TRAIL receptor agonists. Here we report that metformin sensitizes TNBC cells to TRAIL receptor agonists in vitro and in vivo. Metformin selectively

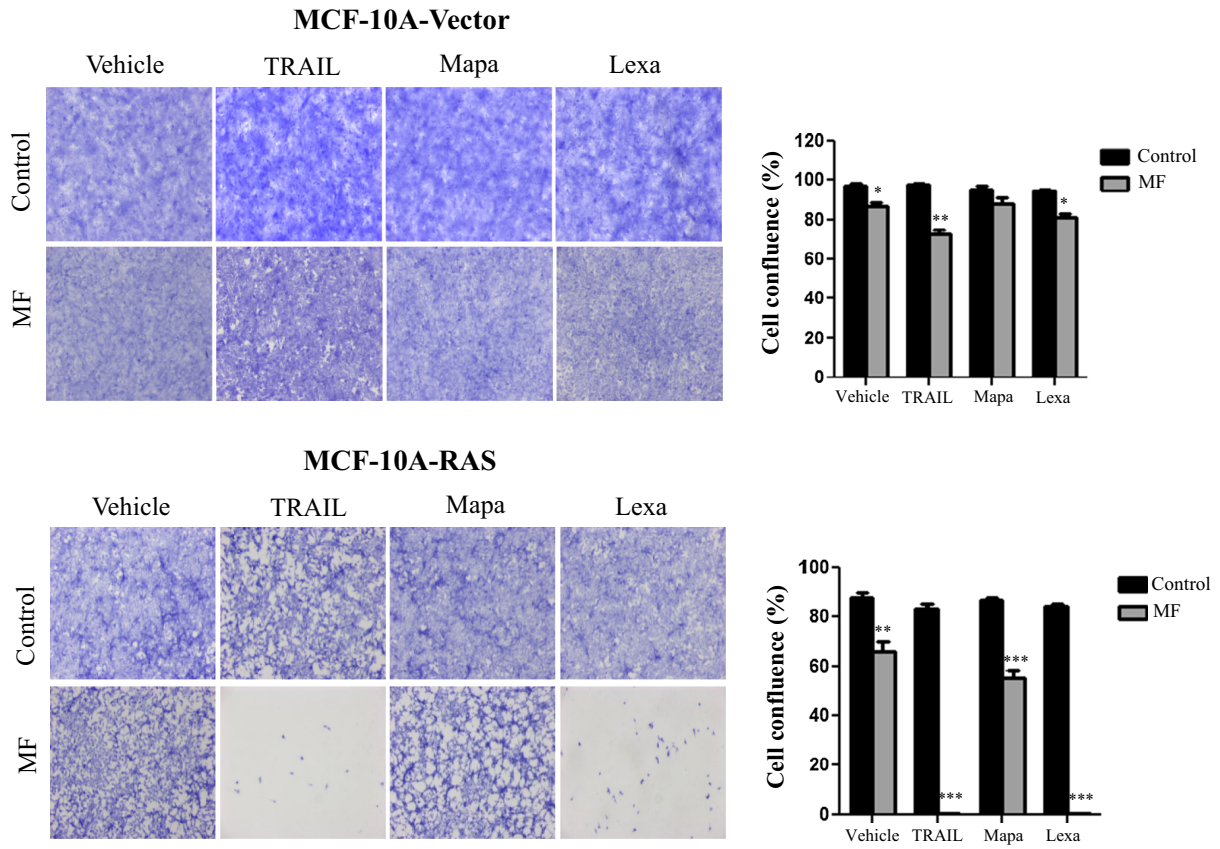


Fig. 2 Metformin sensitizes transformed breast epithelial cells to TRAIL receptor agonists. Crystal violet cell survival assay of MCF-10A-Vector or MCF-10A-Ras cells preincubated with vehicle or metformin (5 mM) for 48 h, and then treated with vehicle or TRAIL, mapatumumab, or lexatumumab (each 1.5 μg/ml) for an additional 24 h. *Left panels* representative images. *Right panels* the percentage confluence of crystal violet-positive cells was scored (mean ± SEM, $n = 3$). In all panels, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

enhances the sensitivity of transformed breast epithelial cells to TRAIL receptor agonist-induced caspase activation and apoptosis with little effect on untransformed breast epithelial cells. These effects of metformin are accompanied by a robust reduction in the expression level of the antiapoptotic protein XIAP. Metformin also enhances the antitumor effects of TRAIL in a murine model of TNBC. Collectively, our findings indicate that metformin enhances the clinical activity of TRAIL receptor agonists, and suggests that additional translational studies combining these agents are warranted.

Methods and materials

Cell culture and reagents

Human MDA-MB-231 and GILM2 TNBC cells stably expressing mCherry and parental GILM2 TNBC cells were maintained as described [24]. MDA-MB-468 TNBC cells were cultured in DMEM media supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin (ThermoFisher Scientific/Gibco). Human MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or vector were maintained as described [25]. Mapatumumab and lexatumumab were kindly provided by Dr. Robin Humphreys (Human Genome Sciences). Recombinant TRAIL peptide (amino acids 95–281) was purified as described [11].

Crystal violet cell survival assay

A crystal violet cell survival assay was performed as described (10). Cells were seeded on 6-well plates (3×10^5 cells/well) overnight. Cells were then preincubated with vehicle or metformin (1 mM for MDA-MB-468 cells or 5 mM for MDA-MB-231-mCherry and GILM2 cells) for 48 h, and treated with vehicle or TRAIL, mapatumumab, or lexatumumab (each 1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for an additional 24 h (MDA-MB-231-mCherry and GILM2 cells) or 48 h (MDA-MB-468 cells) before staining with crystal violet. The percentage cell confluence of crystal violet-positive cells was determined in 3 fields per treatment condition using NIH ImageJ software. Cells in each field were colored using "color threshold." Percentage confluence of colored cells was quantified using "analyze particles" which reports the percent area in each field occupied by colored cells.

Immunoblotting

Whole-cell lysates were immunoblotted as described [25] using primary Abs against XIAP, β-actin (Sigma-Aldrich), PARP (BD Biosciences), and procaspase-3 (Cell Signaling Technology).

Fig. 3 Metformin enhances TRAIL-induced caspase activation in TNBC cells. **a** Immunoblots of TNBC cells preincubated with vehicle or metformin (5 mM) for 48 h and then treated with vehicle or TRAIL (1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for 16 h. **b** TNBC cells were preincubated with vehicle or metformin (5 mM) for 48 h, treated with vehicle or TRAIL (1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for 24 h, and caspase-3/7 activity was measured (mean ± SEM, n = 3). **c** Immunoblots of MCF-10A breast epithelial cells stably expressing vector or H-RasV12 preincubated with vehicle or metformin (5 mM) for 48 h and then treated with vehicle or TRAIL (1.5 μg/ml) for 16 h. **d** MCF-10A-Vector or MCF-10A-RAS cells were preincubated with vehicle or metformin (5 mM) for 48 h, treated with vehicle or TRAIL (1.5 μg/ml) for 24 h, and caspase-3/7 activity was measured (mean ± SEM, $n = 3$). In **b**, **d** $^{**}P < 0.01$ and $^{***}P < 0.001$

Caspase-3/7 activity assay

The Caspase-Glo 3/7 Assay System (Promega) was used to measure caspase-3/7 activity in cell lysates according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates (2.5×10^3 cells/well) overnight. The next day, cells were preincubated with vehicle or metformin (5 mM) for 48 h and then treated with vehicle or TRAIL (1.5 μg/ml for MDA-MB-231-mCherry, GILM2, MCF-10A and MCF-10A-RAS cells, or 4 μg/ml for MDA-MB-468 cells) for an additional 24 h. Caspase-3/7 activity was normalized to cell number and expressed as fold activity compared to vehicle-treated cells.

TRAIL receptor cell surface expression

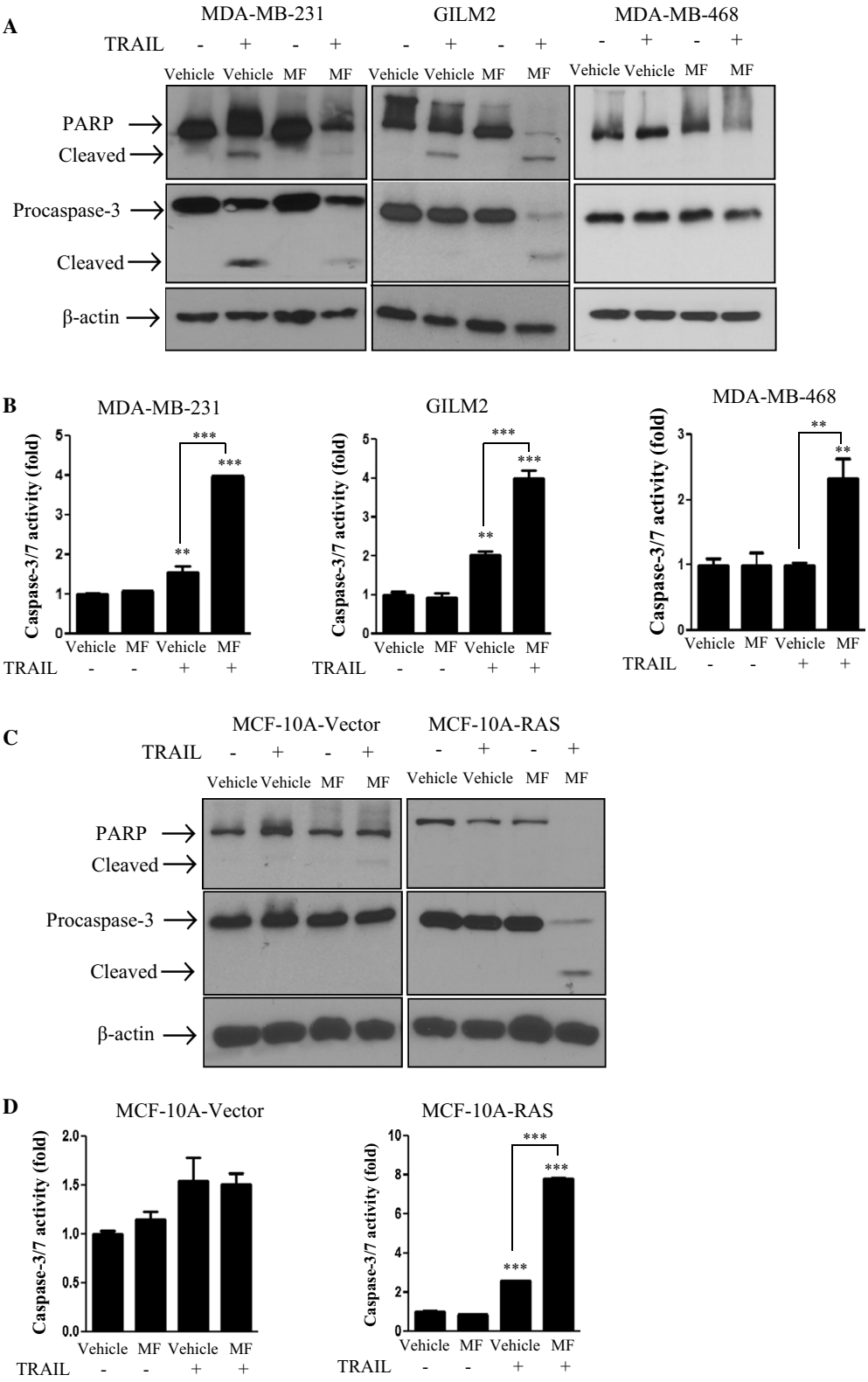
Cell surface expression of TRAIL receptors (TRAIL-R1 and TRAIL-R2) was determined by flow cytometry using TRAIL-R1, TRAIL-R2, or control IgG1 mAb conjugated with allophycocyanin (BioLegend) as described previously [14].

XIAP siRNA experiments

siRNAs targeting the sequences GAAGCUAGAUUAAAG UCCU (si1-XIAP) or CAGUGAAGACCCUUGGGAA (si2-XIAP) in human XIAP and non-silencing control siRNA were purchased from Sigma-Aldrich. Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (ThermoFisher Scientific) according to the manufacturer's protocol.

Real-time PCR

Real-time PCR for TRAIL-R1, TRAIL-R2, and GAPDH was performed as described previously [14]. Primers for XIAP (forward 5-AGTGCCACGCAGTCTACAAA,


A
MDA-MB-231
GILM2
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TRAIL
Vehicle
MF
PARP
Cleaved
Procaspase-3
β-actin
B
MDA-MB-231
GILM2
MDA-MB-468
Caspase-3/7 activity (fold)
C
MCF-10A-Vector
MCF-10A-RAS
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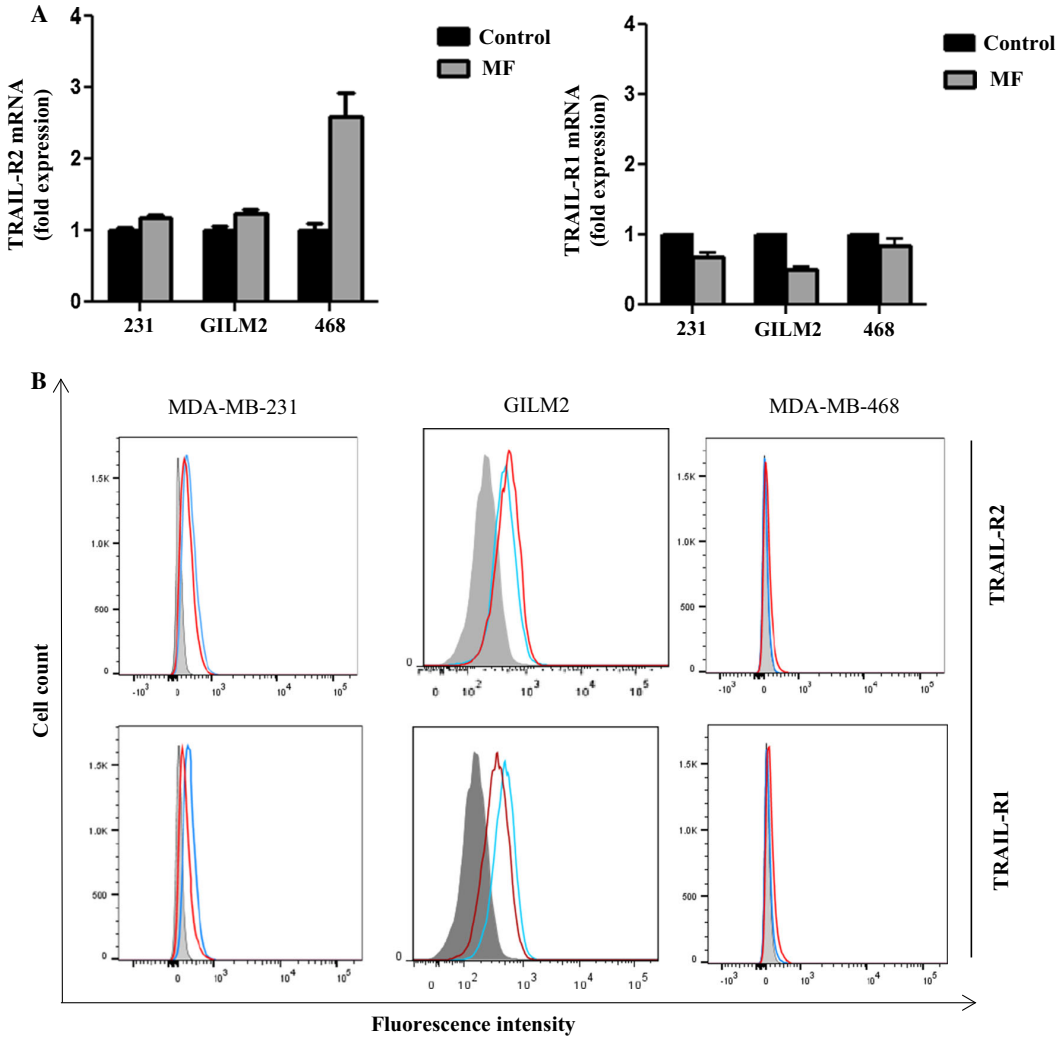


Fig. 4 Metformin does not alter cell surface expression of TRAIL receptors in TNBC cells. **a** TNBC cells were treated with vehicle or metformin (5 mM) for 72 h. TRAIL-R2 (*left panel*) and TRAIL-R1 (*right panel*) mRNA levels were determined by real-time PCR and were normalized to expression in vehicle-treated TNBC cells. **b** TNBC cells were treated with vehicle or MF (5 mM) for 72 h and then incubated with IgG, TRAIL-R1, or TRAIL-R2 Ab. Cell surface expression of TRAIL receptors was determined by flow cytometry. *Gray bar* TNBC cells incubated with IgG. *Blue line* TNBC cells treated with vehicle and incubated with TRAIL-R1 (*bottom panels*) or TRAIL-R2 (*top panels*) Ab. *Red line* TNBC cells treated with metformin and incubated with TRAIL-R1 (*bottom panels*) or TRAIL-R2 (*top panels*) Ab

reverse 5-GCATGTGTCTCAGATGGCCT) were purchased from Integrated DNA Technologies and real-time PCR was performed using the same methods. A comparative Ct method was used to normalize RNA expression in samples to the controls in each experiment.

Orthotopic model of metastatic TNBC

GILM2-mCherry TNBC cells (2×10^6) were resuspended in Matrigel (BD Biosciences) and injected bilaterally into the 4th mammary gland ducts of 4- to 5-week-old female NOD *scid* IL2 receptor γ chain knockout (NSG) mice (Jackson Laboratory). Mice were randomized into four treatment groups (10 mice per group) three weeks after tumor inoculation: (1) PBS vehicle *i.p.* daily; (2) metformin 2 mg/ml in the drinking water; (3) TRAIL (10 mg/kg *i.p.* daily), or (4) metformin (2 mg/ml in the drinking water) plus TRAIL (10 mg/kg *i.p*. daily). Mammary tumor volume was calculated as described [26]. Lung metastases were visualized by fluorescence microscopy in isolated whole lungs and scored using NIH ImageJ analysis as described [26]. All animal experiments were carried out as

part of an IACUC-approved protocol at the University of Wisconsin–Madison.

Tumor apoptosis assay

Formalin-fixed, paraffin-embedded tumor tissue sections were analyzed for active caspase-3 expression by immunohistochemistry using an Ab against cleaved caspase-3 (Cell Signaling) as described [26].

Statistics

The statistical significance of differences between groups was determined by ANOVA with Bonferroni post tests using GraphPad Prism 4 software.

Results

Metformin sensitizes TNBC cells to TRAIL receptor agonists

To determine whether metformin sensitizes TNBC to TRAIL receptor agonists, three human TNBC cell lines (MDA-MB-231-mCherry, GILM2, and MDA-MB-468) were preincubated with vehicle or metformin and then treated with TRAIL, mapatumumab, or lexatumumab. Metformin sensitized all three TNBC cell lines to TRAIL receptor agonists, with the most robust effects observed in MDA-MB-231-mCherry and GILM2 cells. (Figure 1). A dose–response experiment in MDA-MB-468 cells identified 0.5 mM metformin as the minimal concentration needed to sensitize these TNBC cells to TRAIL receptor agonists (Fig. S1). In addition, preincubation with metformin sensitized human HT29 colon adenocarcinoma and DU145 prostate cancer cell lines to TRAIL receptor agonists (Fig. S2). Collectively, these results demonstrate that metformin augments the cytotoxicity of TRAIL receptor agonists against a broad range of tumor cell types, including TNBC cells.

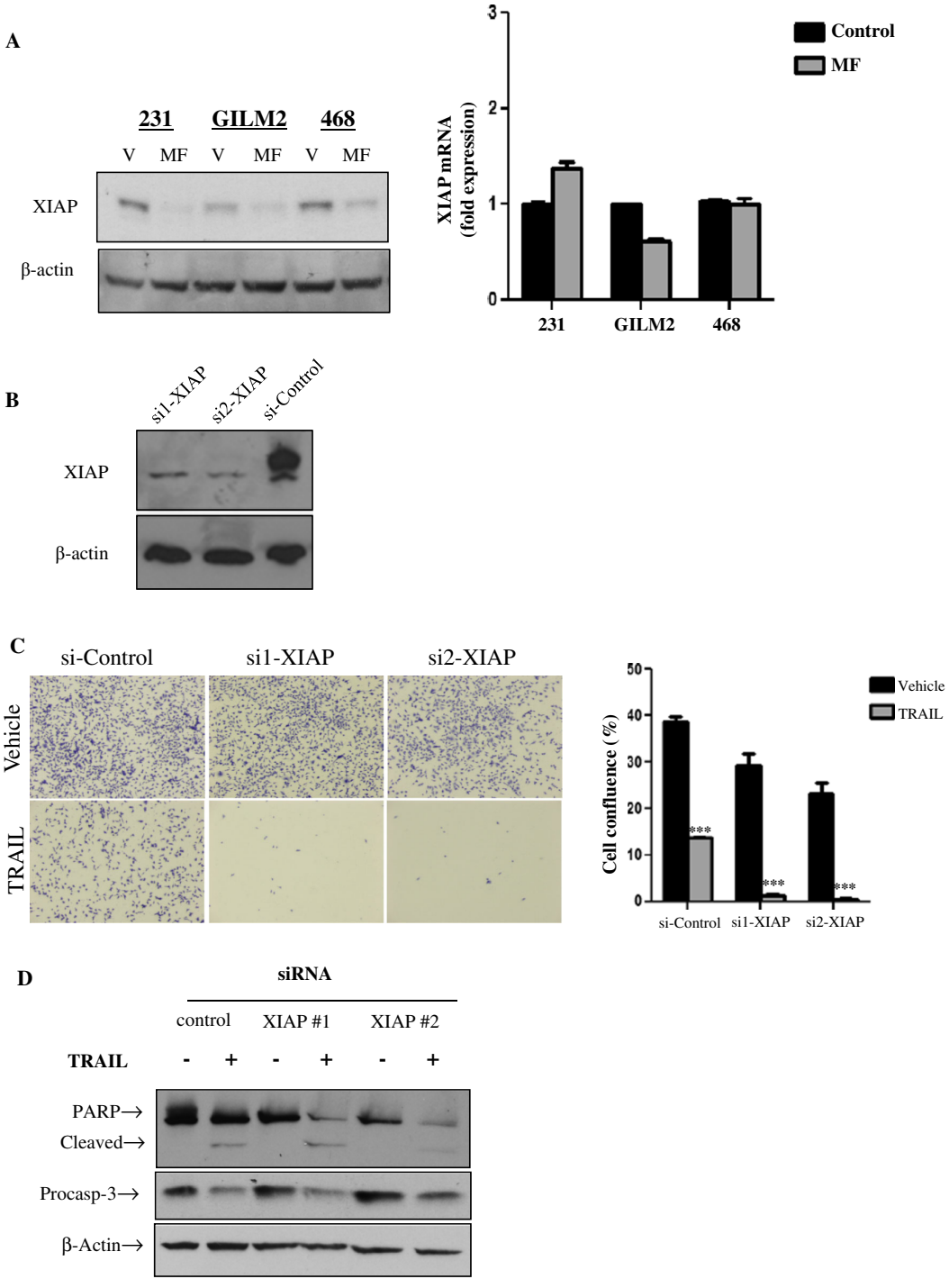
Metformin sensitizes transformed breast epithelial cells to TRAIL receptor agonists

To determine whether metformin enhanced the cytotoxicity of TRAIL receptor agonists preferentially in transformed cells, we utilized human MCF-10A breast epithelial cells transformed by oncogenic H-RasV12 and untransformed MCF-10A cells stably expressing empty vector. Strikingly, metformin robustly sensitized MCF-10A-Ras cells to TRAIL and lexatumumab, but had a minimal effect on the sensitivity of untransformed MCF-10A-Vector cells to these agents (Fig. 2). In contrast, metformin had only a modest effect on the cytotoxicity of mapatumumab against MCF-10A-Ras cells. Collectively, these results indicate that metformin preferentially sensitizes transformed cells to TRAIL receptor agonists and support the potential tumor selectivity of this therapeutic combination.

Fig. 5 Metformin reduces XIAP protein expression in TNBC cells and silencing XIAP sensitizes TNBC cells to TRAIL. **a** Immunoblot of XIAP protein levels (*left panel*) and real-time PCR analysis of XIAP mRNA levels (*right panel*) in TNBC cells treated with vehicle or metformin (5 mM) for 72 h (immunoblots) or 48 h (real-time PCR). **b** MDA-MB-231-mCherry cells were transfected with a non-silencing control siRNA (si-Control) or one of two different siRNAs targeting XIAP (si1-XIAP or si2-XIAP). Immunoblot of XIAP expression 48 h after transfection. **c** Crystal violet cell survival assay of MDA-MB-231-mCherry cells transfected with control or XIAP siRNAs, and 48 h later treated with vehicle or TRAIL (0.3 μg/ml) for 24 h. *Left panel* representative images. *Right panel* quantification of cell confluence in 3 fields for each treatment (mean ± SEM, $n = 3$) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **d**, Silencing XIAP enhances TRAIL-induced caspase activation in TNBC cells. MDA-MB-231-mCherry cells were transfected with control or XIAP siRNAs, and 48 h later were treated with vehicle or TRAIL (0.3 μg/ml) for 16 h. PARP (full-length and caspase-cleaved) and procaspase-3 levels were determined by immunoblotting

Metformin enhances TRAIL-induced caspase activation in TNBC cells

To investigate whether metformin promotes TRAIL-induced caspase activation, TNBC cells were preincubated with metformin, treated with vehicle or TRAIL, and then analyzed by immunoblotting. Metformin promoted TRAIL-induced proteolysis of the caspase substrate PARP as detected by diminished full-length PARP and/or increased cleavage product compared to vehicle-treated cells (Fig. 3a). In addition, metformin enhanced TRAIL-induced procaspase-3 proteolytic processing as detected by decreased procaspase-3 levels and/or increased cleaved subunit compared to vehicle-treated cells. Similarly, pretreatment of HT29 and DU145 carcinoma cells with metformin augmented TRAIL-induced caspase activation (Fig. S3). To quantitate more precisely the effects of metformin and TRAIL on caspase activation, we utilized a caspase-3/-7 activity assay. Metformin robustly enhanced TRAIL-induced caspase-3/-7 activity in all three TNBC cells compared to cells treated with either agent alone (Fig. 3b). Metformin also enhanced TRAIL-induced caspase activation in MCF-10A-Ras cells, while untransformed MCF-10-Vector cells were not sensitive to this combination (Fig. 3c, d). Consistent with its reported mechanism of action [18, 19], metformin increased p-AMPK levels and selectively inhibited mTORC1 as determined by reduced phosphorylation of the mTORC1


A
231
GILM2
468
V
MF
V
MF
V
MF
XIAP
β-actin
XIAP mRNA (fold expression)
Control
MF
231
GILM2
468
B
si1-XIAP
si2-XIAP
si-Control
XIAP
β-actin
C
si-Control
si1-XIAP
si2-XIAP
Vehicle
TRAIL
Cell confluence (%)
Vehicle
TRAIL
si-Control
si1-XIAP
si2-XIAP
D
siRNA
control
XIAP #1
XIAP #2
TRAIL
PARP→
Cleaved→
Procasp-3→
β-Actin→

substrate p-S6 and no effect on the mTORC2 substrate p-Akt (Fig. S4). Taken together, these findings indicate that metformin potently augments caspase activation and apoptosis by TRAIL in a broad range of tumor cell types and provide additional evidence for the tumor selectivity of this combination.

Metformin does not alter cell surface expression of TRAIL receptors in TNBC cells

To determine whether metformin sensitizes cancer cells to TRAIL by increasing the expression of its proapoptotic receptors (TRAIL-R1 and TRAIL-R2), TNBC cells were treated with metformin and TRAIL receptor mRNA levels were measured by real-time PCR. Metformin treatment resulted in a modest increase in TRAIL-R2 mRNA levels in MDA-MB-468 TNBC cells, but had little effect on TRAIL-R2 levels in the other TNBC cell lines or on TRAIL-R1 mRNA levels in any of the TNBC cell lines (Fig. 4a). Furthermore, metformin did not significantly affect cell surface expression of either proapoptotic TRAIL-R1 or TRAIL-R2 receptors as determined by flow cytometry (Fig. 4b). Metformin also did not alter protein levels of MAGED2 (Fig. S5), which we previously demonstrated to be downregulated by methionine restriction [14]. These findings indicate that the TRAIL-sensitizing effects of metformin are not due to enhanced TRAIL receptor expression or cell surface localization in TNBC cells.

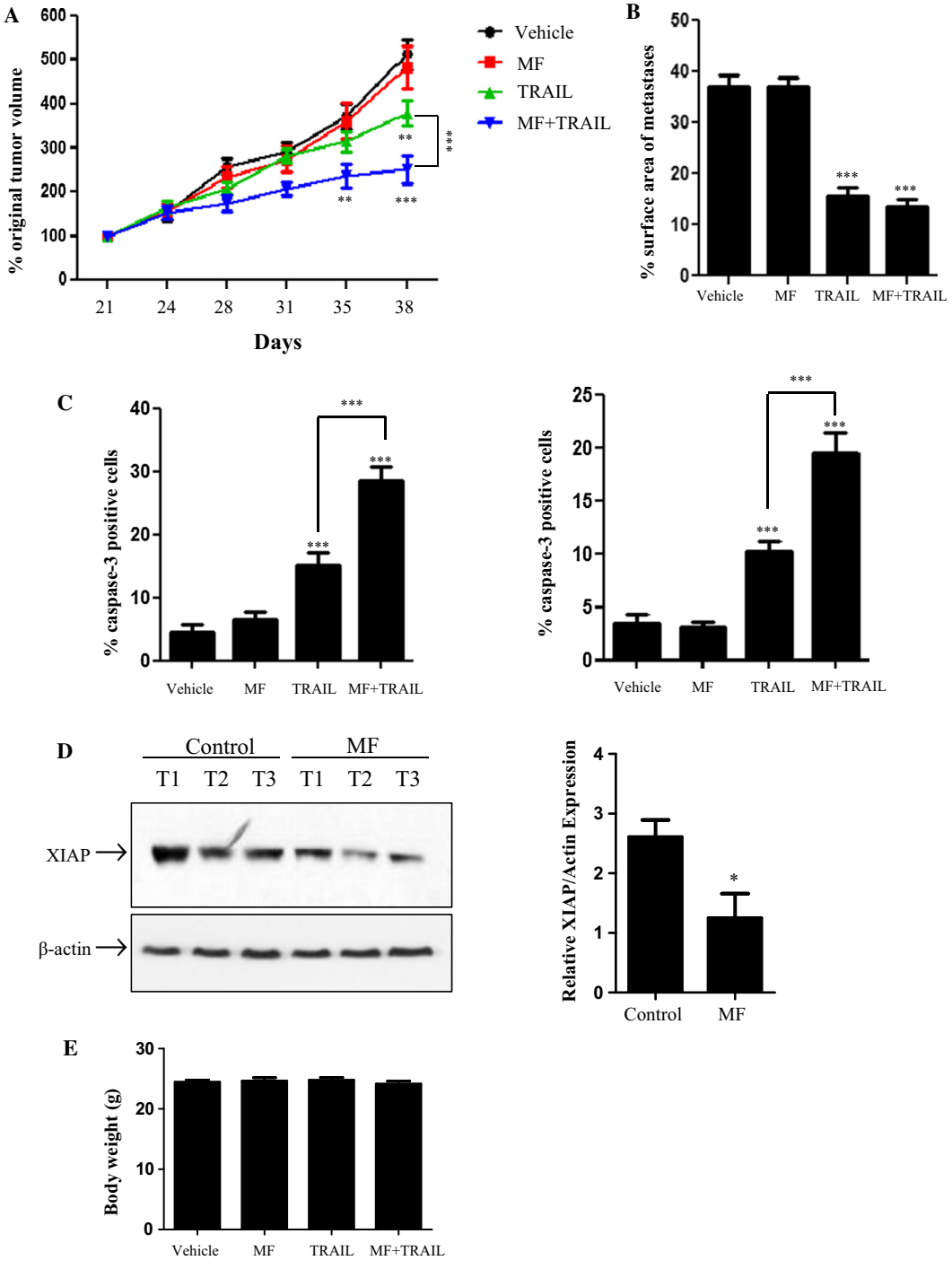
Metformin reduces XIAP expression in TNBC cells and silencing XIAP sensitizes TNBC cells to TRAIL

The antiapoptotic X-linked inhibitor of apoptosis protein (XIAP) has been demonstrated to confer resistance to TRAIL-induced apoptosis by suppressing caspase activation [27, 28]. Therefore, we postulated that metformin might sensitize cancer cells to TRAIL by downregulating XIAP. Consistent with this hypothesis, treatment of MDA-MB-231-mCherry, GILM2, and MDA-MB-468 TNBC cells with metformin resulted in a reduction in XIAP protein levels (Fig. 5a, left panel). In contrast, treatment with metformin did not affect XIAP mRNA levels (Fig. 5a, right panel), indicating that metformin regulates XIAP via a posttranscriptional mechanism. To examine the functional role of XIAP downregulation in the TRAIL-sensitizing effects of metformin, MDA-MB-231 cells were transfected with a scrambled siRNA (si-Control) or one of two different siRNAs targeting XIAP (si-1 XIAP and si-2 XIAP). Both siRNAs targeting XIAP reduced XIAP protein levels compared to the scrambled control siRNA (Fig. 5b). Notably, silencing XIAP in MDA-MB-231-mCherry cells had a modest effect on cell viability and robustly sensitized these cells to TRAIL treatment compared to a scrambled control siRNA (Fig. 5c). To examine whether silencing XIAP enhanced TRAIL-induced caspase activation, MDA-MB-231-mCherry cells were transfected with siRNAs targeting XIAP or a scrambled control and then treated overnight with vehicle or TRAIL. Silencing XIAP enhanced TRAIL-induced cleavage of the caspase substrate PARP (reduction of full-length PARP and/or increased cleavage product), while levels of procaspase-3 proteolysis (reduction of procaspase-3 levels) were comparable in TRAIL-treated cells transfected with scrambled siRNAs or siRNAs targeting XIAP (Fig. 5d). Collectively, these results indicate that metformin sensitizes TNBC cells to TRAIL-induced apoptosis by downregulating expression of XIAP.

Fig. 6 Metformin enhances the antitumor effects of TRAIL in an orthotopic model of metastatic TNBC. Three weeks after intraductal injection of tumor cells, female NSG mice with GILM2-mCherry mammary tumors were randomized into 4 groups (10 mice per group): control group (PBS *i.p.* daily for three weeks), metformin (2 mg per ml in drinking water for three weeks), TRAIL (10 mg/kg *i.p.,* daily for three weeks), or metformin plus TRAIL (same dosing as in single-agent arms). **a** The percentage of the original mammary tumor volume (at 3 weeks) in each group (mean ± SEM, $n = 10$ mice per group). **b** The percentage of the surface area occupied by lung metastases (mean ± SEM, $n = 10$ mice per group). **c** The percentage active caspase-3-positive tumor cells in mammary tumors (*left panel*) or metastatic lung tumors (*right panel*) after treatment (mean ± SEM, $n = 3$ tumors per group). **d** Immunoblot of XIAP expression in mammary tumors from control vehicle-treated or metformin-treated mice ($n = 3$ mammary tumors per group). XIAP expression normalized to β-actin was measured by Image J analysis. **e** Body weight of the mice in each treatment group at the end of the study (mean ± SEM, $n = 10$ mice per group). In all panels, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated mice or the indicated comparison

Metformin enhances the antitumor effects of TRAIL in an orthotopic model of metastatic TNBC

To determine whether metformin augments the antitumor effects of TRAIL in an orthotopic model of metastatic TNBC, we treated female NSG mice bearing established GILM2-mCherry mammary tumors with vehicle, metformin alone, TRAIL alone, or the combination of metformin and TRAIL. Under the conditions tested, metformin had no significant effect on mammary tumor growth or lung metastases. In contrast, TRAIL inhibited mammary tumor growth, but the combination of TRAIL and metformin was more effective than TRAIL alone (Fig. 6a). Both therapies, TRAIL alone or in combination with metformin, inhibited lung metastases to a comparable degree (Fig. 6b). Moreover, both TRAIL alone or in combination with metformin induced apoptosis in mammary tumors and lung metastatic lesions as determined by


A
% original tumor volume
Days
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MF
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MF+TRAIL
B
% surface area of metastases
C
% caspase-3 positive cells
D
Control
MF
T1 T2 T3 T1 T2 T3
XIAP
β-actin
Relative XIAP/Actin Expression
E
Body weight (g)

active, cleaved caspase-3 immunostaining (Fig. 6c). Consistent with our in vitro findings, the combination of TRAIL and metformin resulted in more apoptosis induction in mammary tumors and lung metastases than either TRAIL or metformin alone. Importantly, metformin treatment reduced XIAP protein levels in mammary tumors (Fig. 6d), consistent with our in vitro findings. None of the treatments affected the body weight of the mice at the end of the study compared to control vehicle-treated mice (Fig. 6e). Collectively, these findings indicate that metformin enhances the antitumor activity of TRAIL in vivo and provide preclinical evidence supporting additional translational studies investigating the combination of metformin and TRAIL receptor agonists in metastatic TNBC.

Discussion

Based largely on epidemiologic data and promising preclinical studies, the oral diabetes medication metformin has been incorporated into a multitude of clinical trials in diverse early-stage and advanced malignancies, either as monotherapy or in combination with cytotoxic agents or radiation [19]. We have demonstrated that metformin sensitizes TNBC cells to proapoptotic TRAIL receptor agonists, while untransformed breast epithelial cells are largely resistant to this combination. Although other groups have recently demonstrated that metformin enhances TRAIL-induced apoptosis in cultured cancer cells [29–32], we have provided the first in vivo evidence for the therapeutic utility of this combination in an orthotopic model of metastatic TNBC that recapitulates many features of the human disease [24]. Specifically, we showed that metformin augments the antitumor effects of TRAIL against mammary tumors and increases apoptosis in mammary tumors and lung metastases compared to treatment with either agent alone. Importantly, these antitumor effects in vivo were observed at metformin doses that were well tolerated by the mice, a critical point because the TRAIL-sensitizing effects of metformin in vitro typically require millimolar concentrations of metformin in standard cell culture media due to their supraphysiologic glucose concentration [33, 34]. Given the lack of efficacy of TRAIL receptor agonists in clinical trials in advanced solid tumors [3–8], our findings are particularly significant from a translational perspective because they suggest that metformin may enhance the efficacy of TRAIL receptor agonists in clinical trials. As such, our results provide the first murine model evidence for the clinical utility of the combination of metformin and TRAIL receptor agonists, thereby providing critical preclinical evidence to support additional translational studies in poor prognosis of TNBC, which currently lack targeted therapies [35].

Indeed, metformin is a particularly attractive TRAIL-sensitizing agent based on its well-documented safety in both diabetic and non-diabetic patients, its low cost, and its beneficial impact on metabolic health by improving insulin sensitivity [18, 19]. In addition, our results indicate that the combination of metformin and TRAIL receptor agonists activates apoptosis in p53 mutant TNBC cells and preferentially induces apoptosis in transformed breast epithelial cells with little effect on untransformed breast epithelial cells. Although the mechanism of this tumor selectivity remains poorly understood, our findings suggest that the toxicity of the combination therapy may be modest. Moreover, our studies may provide insights into the reduced cancer incidence in individuals treated with metformin reported in many epidemiologic studies [19–22]: metformin may enhance the sensitivity of nascent tumors to TRAIL-dependent immune surveillance pathways.

Mechanistically, we have demonstrated that metformin sensitizes TNBC cells to TRAIL receptor agonists by downregulating the antiapoptotic protein XIAP. XIAP is a direct inhibitor of caspases that confers resistance to TRAIL-induced caspase-3 activation and apoptosis, while induction of TRAIL-induced apoptosis requires XIAP to be displaced from caspases by mitochondrial release of Smac/DIABLO [27, 28]. We observed that metformin reduces protein expression of XIAP but does not alter XIAP mRNA levels, thereby pointing to a posttranslational mechanism. Intriguingly, rapamycin was previously demonstrated to negatively regulate protein translation of XIAP [36], suggesting that metformin may reduce XIAP protein levels via inhibition of mTORC1. Moreover, metformin treatment reduced XIAP protein levels in mammary tumors in our murine model of TNBC, indicating that the dosing used in our study was sufficient to target this pathway in vivo. To determine the functional relevance of the downregulation of XIAP protein by metformin, we used RNAi and observed that XIAP silencing robustly sensitizes TNBC cells to TRAIL-induced caspase activation and cell death, underscoring the importance of this molecular event in sensitizing TNBC cells to TRAIL. Our results are also consistent with a prior report demonstrating that a small molecule inhibitor of XIAP (embelin) sensitizes inflammatory breast cancer cells lines to TRAIL [37].

Notably, we did not observe any significant effects of metformin on the cell surface expression of TRAIL receptors in contrast to other reports that metformin increases TRAIL-R2 protein levels, although the cellular localization of the receptor was not delineated in these prior studies [29, 31]. Additionally, metformin did not affect protein levels of MAGED2, indicating that

metformin sensitizes TNBC cells to TRAIL receptor agonists by a different mechanism than methionine restriction, which increases cell surface expression of TRAIL-R2 by downregulating expression of MAGED2, an inhibitor of TRAIL receptor expression [14, 38]. Furthermore, other groups have implicated downregulation of the antiapoptotic protein FLIP as an important molecular event in the TRAIL-sensitizing effects of metformin [12, 39]. Although our results do not exclude the potential contribution of FLIP or other molecular targets, our studies point to the downregulation of XIAP by metformin as a functionally relevant event for its TRAIL-sensitizing effects.

In conclusion, we have demonstrated that metformin downregulates XIAP and sensitizes clinically aggressive TNBC cells to TRAIL receptor agonists. We have also shown that the combination of metformin and TRAIL has robust antitumor activity in a murine model of metastatic TNBC which recapitulates features of the human disease. Given the lack of targeted therapies for TNBC and the largely disappointing activity of TRAIL receptor agonists in clinical trials to date, our results point to the combination of metformin and TRAIL receptor agonists as a promising approach to enhance activity of these proapoptotic agents. Finally, our studies suggest that inhibition of XIAP expression by metformin may be a useful biomarker to predict response to this combined therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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Title: **Synthetic lethal metabolic targeting of senescent cells after androgen deprivation therapy**

1. Introduction

Progression to castrate resistant prostate cancer for men with advanced prostate cancer (PC) results after the initiation of androgen deprivation therapy (ADT). One underutilized therapeutic strategy that has the potential to dramatically improve outcomes is eradicating the persistent cancer cells that remain after ADT and likely play a key role in the development of castration-resistant PC. The initiation of ADT induces susceptibilities in PC cells that make them amenable to synergistic treatment and improved cell killing.

Androgen withdrawal in murine xenografts and human PC tissues is associated with a decrease in the proliferative index, but surprisingly low levels of apoptosis. We and others have demonstrated that a substantial portion of these persistent cells express markers of cellular senescence, a terminal growth arrest characterized by exit from the cell cycle and senescence-associated β-galactosidase expression. These senescent cells, although not proliferating, generate a protumor response, the senescent secretory phenotype, that may be detrimental to the patient and must be removed. However, the unique metabolic phenotype expressed by these persistent senescent cells is characterized by increased protein synthesis and notably an amplified proteotoxic stress response (PSR), a conserved survival pathway characterized by induction of multiple heat shock protein (Hsp) families coordinated by the master transcriptional regulator Hsf1. It is our overall hypothesis that the new senescent phenotype induced in prostate cancer cells by ADT may result in unique vulnerabilities to drugs targeting pathways such as the PSR that are critical for survival in the senescent state.

In preliminary data activation of the PSR in these residual cancer cells may represent a pathway critical for the survival of senescent PC cells. Further experiments have identified one agent, metformin, a widely-used, nontoxic oral antidiabetic drug that we propose to repurpose as synthetic lethal therapy in combination with ADT. We postulate that that metformin is synthetic lethal with ADT because it disables the principal PSR pathway mediated by Hsf1 in senescent PC cells that are already experience high levels of proteotoxic stress.

In Aim 1 we will examine the activity of metformin in eradicating senescent PCs following ADT in cellular models. In addition, we will determine whether metformin's actions are specifically mediated by inactivation of Hsf1 and resultant disruption of the PSR cell survival pathway mediated by Hsp27, Hsp70 and Hsp 90. In vitro and xenograft PC models utilizing overexpression of a phosphorylation-resistant Hsf1 mutant will be used to interrogate the specific role of the HSf1-mediated PSR in the synthetic lethal response. PC has a variable response to ADT. The ability of metformin to clear senescent cancer cells after ADT will be examined in Aim 2 in a series of human PC xenografts that exhibit variable responses to ADT. We will utilize a xenograft system consisting of human prostate cancer tumors that can be exposed to drug combinations in a physiologically relevant setting, the growth easily tracked, and the tumor readily harvested for detailed examination. Experiments will test whether synchronous ADT-metformin or their stepwise use leads to better tumor regression and longer survival. In addition, markers of response will be investigated in the tumors focusing on the Hsps examined in Aim 1. Finally, in Aim 3 we will employ a health sciences research approach using the National Department of Veterans Affairs Corporate Data Warehouse to investigate a retrospective cohort of patients on ADT (~260,000 men), 8% of whom are on metformin (~21,000), to determine

PC-specific mortality, biochemical recurrence-free survival and skeletal related events. This will provide further evidence for the implementation of this novel synthetic lethal therapeutic strategy.

Our studies have the potential to lead to a new treatment paradigm for PC by specifically targeting a unique vulnerability of senescent PCs (the PSR) that persist following ADT and likely contribute to androgen-resistance. The proposed study directly addresses mechanisms of resistance for men with high-risk cancer and furthermore, since metformin may mitigate the metabolic side effects of ADT, may improve the physical health of men with PC. When completed our new synthetic lethal approach to PC can be readily translated into the clinic since both ADT and metformin are safe and currently in use.

2. Keywords

prostate cancer, androgen deprivation therapy, senescence, proteotoxic stress, xenograft models, metformin, synthetic lethality

3. Accomplishments

SPECIFIC AIM 1:

Major Task 1: Determine whether the ADT-metformin synergistic response is mediated via disruption of the Hsf1-mediated proteolytic stress response (PSR).

Subtask 1: Characterize the effects of metformin on the viability of senescent PC cells following ADT (Jarrard/Cryns)

- PC cells will be treated with vehicle or biculatamide for 4 days followed by metformin or vehicle for 2-4 days
- Score senescent PC cells using SA-β-gal activity, GLB1 immunostaining and flow cytometry
- Evaluate apoptosis using co-immunofluorescence with GLB1 and active caspase-3 Ab and by annexin V labeling of GLB1-flourescent PC cells
- Cell lines: LNCaP, CWR22Rv1, VCaP in subtasks 1-3

Ongoing. We have optimized dosing and time to achieve a maximal coefficient index for synthetic lethality for 3 cell lines, LNCaP, CWR22Rv1 and LAPC4. VCaP was not utilized because of variable androgen responsiveness. LAPC4 was used as an alternate.

Senescence has been quantitated using SA-β-gal activity, and flow cytometry as well as western for p16 and p27. Apoptosis occurs maximally at 48 hr after exposure to metformin as noted in the cell line LAPC4 Figure 1.

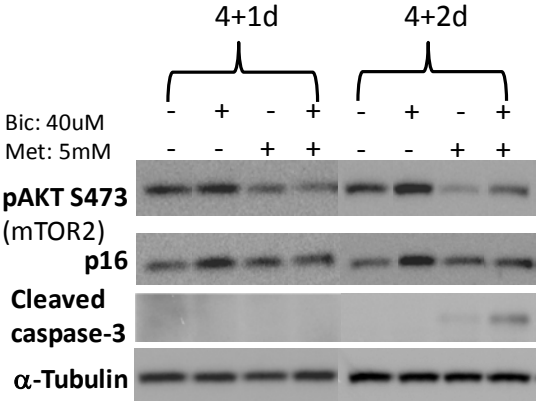

Figure 1 legend: LAPC4 Cells were treated with bicalutamide for 4 days, and then bicalutamide was removed followed by treatment of metformin for 1 or 2 days. Induction of p16 is a senescent marker. CC3 indicates increased apoptosis.

Senescence occurs in a subpopulation and we are currently investigating options for selecting cells. Sorting for SA-B-gal expression is too laborious and not optimal. We also attempted to flow sort based on size but this approach is too slow. We are currently using senescence surface marker to isolate senescent cells. The expression of plasma membrane-associate proteins VSP26A, DCR2 and B2M increased in senescent cells, we are using biotinylated antibodies against these proteins, capturing with streptavidin magnetic beads, and isolating with a microchamber.

Subtask 2: Characterize the effects of metformin on the PSR of senescent PC cells following ADT (Cryns/Jarrard).

- PC cells will be treated with ADT and/or metformin as in subtask 1
- Collect GLB1-fluorescent and non-fluorescent cells by FACs and determine Hsf1, Hsp27, Hsp70 and Hsp90 mRNA and protein levels.
- Perform co-immunofluorescence with GLB1 and Hsf1, Hsp27, Hsp70, Hsp90, p-AMPK and AMPK
- Determine whether AMPK binds directly to Hsf1 by co-immunoprecipitation.

Milestones: We predict that metformin will increase binding of AMPK to Hsf1 and inhibit the PSR.

Ongoing. We have examined the proteotoxic stress response in multiple prostate cancer cell lines in response to androgen deprivation therapy with and without metformin treatment. Analyses of the entire population of treated prostate cancer cells has yielded variable results depending on prostate cancer cell line and individual heat shock proteins with regard to the effects of both androgen deprivation and metformin treatment. However, these studies did not specifically examine the effects of these interventions on the subpopulation of senescent prostate cancer cells due to technical difficulties noted in Subtask 1. Using the modified approach outlined in Subtask 1, we will isolate the senescent subpopulation after treatment, and we expect to complete these studies in year 2 of the project. Figure 2.

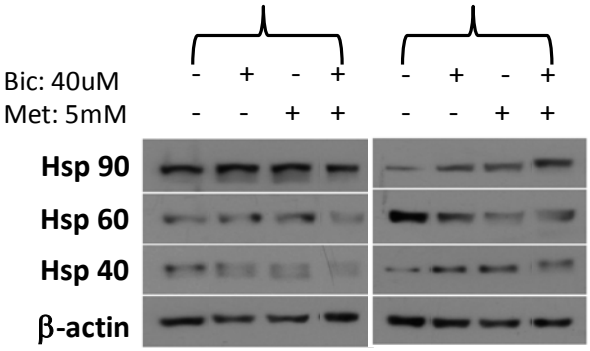

Figure 2 legend: LAPC4 Cells were treated with bicalutamide for 4 days, and then bicalutamide was removed followed by treatment of metformin for 1 or 2 days.

Subtask 3: Determine the functional role of site-specific phosphorylation of Hsf1 on the effects of metformin on senescent PCs following ADT (Cryns/Jarrard).

- Stably transduce PC cells with vector, WT Hsf1 or mutant S121A Hsf1 by lentiviral transduction.

- Treat PC cells stably expressing vector, WT or S121A mutant Hsf1 with ADT and/or metformin as in subtask 1
- Perform cell viability (subtask 1) and molecular characterization (subtask 2) assays on senescent PCs and the entire population of PCs.

Milestones: We predict that the S121A mutant Hsf1 will abrogate the effects of metformin on cell death and PSR following ADT.

Pending.

Subtask 4: Determine the function of site-specific phosphorylation of Hsf1 on the antitumor effects of ADT and metformin in vivo (Jarrard/Cryns).

- Male nude mice with LnCaP and CWR22rv1 flank tumors stably expressing WT or S121A mutant Hsf1 will be randomized to 4 groups (8 mice per group): (1) vehicle + sham operation; (2) vehicle + castration; (3) metformin + sham operation; and (4) metformin + castration. Metformin will be tried simultaneously (groups 3 and 4) or sequentially (groups 5 and 6). Tumor size will be assessed weekly and serum PSA recorded. To assess senescent cell clearing, a parallel experiment will be performed using the same 4 treatment groups (8 mice per group) except that mice will be euthanized 4 weeks after castration and tumors harvested for analysis. (16X(6X3)) for 2 experiments (total 576)
- Mouse tissues will be analyzed for SA-β-gal activity, HP1γ, GLB1, p27, Hsp27, Hsp70, Hsp90, Ki67, active caspase-3 and TUNEL staining

Pending.

Major Task 2: Examine the synthetic lethal response involving ADT-metformin in vivo in cancers of variable androgen sensitivity and test markers of response.

Subtask 1: To determine the optimal schedule for combining ADT and metformin and assess whether metformin eradicates senescent PC cells following ADT (Jarrard). We will specifically determine whether sequential (i.e. ADT then metformin) is superior to simultaneous (ADT and metformin) and whether senescent cells are being cleared from the tumors with the addition of metformin.

- Obtain approval by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC). (Months 1-4)
- Four groups will be randomized using 3 xenografts (58, 23.1 and 81) when flank tumors are 100mm3 to examine the i) Castrated mice (16 animals x 3 xenograft lines), ii) Mice placed on Metformin (mg/kg) given orally daily in intact sham operated mice (16 X 3 xenograft lines), iii) Mice (16 x 3 xenograft lines) will be castrated and placed on Metformin (mg/kg) given orally daily 10d post-castration and iv) Mice (16 x 3 xenograft lines) will be castrated and placed on Metformin (50mg/kg-1) given orally daily immediately post-castration. Post-ADT tumor size will be assessed weekly and serum PSA levels (Total 16X(4X2)) for 2 experiments (total 256). Grand total 832 plus 10% to counter attrition and loss of animals (915).

- Half of the animals (8) in each group will be harvested 4wk after castration to permit an assessment of senescence cell clearing after metformin. The remaining animals will continue to be monitored for survival
- Senescent cell clearing will be measured as described above in 1D using primarily Vectra quantitation of immunohistochemistry. Apoptosis will be examined as described above.

Ongoing studies on track. Animal approval has been obtained and the studies are ongoing. The xenograft lines had to be obtained from Univ Washington and expanded. Test dosing of metformin performed successfully. Currently tumors implanted in experimental mice and tumors growing.

Subtask 2: To determine whether PSR markers predict improved response to ADT-Metformin (Jarrard/Cryns). Xenograft tumors from the 2A will be sectioned and immunofluorescence will be used and quantitated using the automated Vectra™ system for Hsp27, Hsp70 and Hsp90. The proteolytic stress response(PSA) represented by these 3 genes in castrated animals harvested at 4wk (group i) will be statistically compared to tumor response, survival, PSA, and other markers including GLB1 in ADT-Metformin groups (iii and iv).

Pending.

Major Task 3: Determine whether metformin combined with ADT results in improved cancer-specific survival and longer time to secondary interventions in patients on these agents.

Subtask 1: We propose to utilize a robust observational cohort from the national Veterans Affairs (VA) database to specifically evaluate our hypothesis that metformin improves PC response to ADT, thereby directly examining the patient relevance of our preclinical data in validated patient population. Approvals (Jarrard/Richards).

Completed.

Subtask 2: Data collection, organization with exclusion and inclusion from 2000-2008 (Jarrard/Richards).

Completed.

Subtask 3: Analysis of primary and secondary predictive variables (Jarrard/Richards). Evaluate and control for other covariates including other diabetes medication administration history, age, race, Charlson-comorbidity score, agent orange exposure, family history of prostate cancer, tobacco use, blood type, local therapy (surgery or radiation), date of prostate cancer diagnosis, stage at diagnosis, Gleason score, and other medication administration history (finasteride, aspirin, and docetaxol).

Completed.

Milestone(s) Achieved:

Using national Veterans Affairs databases, we identified all men diagnosed with PCa between 2000-2008 that were treated with ADT with follow-up through October of 2015. We excluded patients that were treated with ADT for $\leq$6 months or were receiving ADT concurrently with localized radiation therapy. We split these patients into three cohorts: 1. Patients without diabetes 2. Diabetics on metformin 3.

Diabetics not treated with metformin. Our primary outcome was overall survival (OS) and secondary outcomes included skeletal related events (SRE) and prostate-cancer specific survival. Cox proportional hazards ratios were calculated for overall and disease specific survival.

The total cohort after exclusions consisted of 87,344 patients of which 53,893 (61%) were non-diabetics, 14,517 (17%) were diabetics on metformin, and 18,934 (22%) were diabetics not receiving metformin. The mean age was 75 $\pm$11 years in the non-diabetics, 71 $\pm$12 in the diabetics on metformin, and 75 $\pm$10 in the diabetics no metformin ($p<0.001$). The median OS was 7.1 years in the non-diabetics, 9.1 years in the diabetics on metformin, and 7.4 years in the diabetics not treated with metformin.

Multivariable Cox proportional hazards analysis assessing for predictors of overall survival showed improved survival in diabetics on metformin (HR 0.77, 95% CI 0.74-0.81) vs. diabetics not treated with metformin (HR 0.99, 95% CI 0.95-1.03) with non-diabetics as referent group. Multivariable Cox proportional hazards analysis assessing for predictors of SRE revealed no association between metformin use (HR 0.99, 95% CI 0.92-1.07) and SRE. Lastly, multivariable Cox proportional hazards analysis assessing for predictors of prostate-cancer specific survival showed improved survival in diabetics on metformin (HR 0.72, 95% CI 0.67-0.78) and to a lesser effect diabetics not treated with metformin (HR 0.87, 95% CI 0.81- 0.93) with non-diabetics as referent group.

We conclude that metformin use in Veterans with advanced prostate cancer receiving ADT is associated with improved OS and cancer-specific survival. The impact of metformin in prostate cancer patients should be evaluated in a prospective clinical trial.

Completed and abstract presented at the American Urological Association Meeting May 2017 and the GU ASCO meeting Feb 2017. Paper currently in preparation.

Opportunities for training and professional development?

These include a post-doctoral fellow Dr Shiva Damoradan currently in the laboratory. Additional trainees include Nathan Damaschke a graduate student who performed

How were the results disseminated to communities of interest?

Abstract presentation at the American Urological Association meeting 5/2017 Boston MA.

• What do you plan to do during the next reporting period to accomplish the goals and objectives?

We plan to adhere to the proposed SOW with the exceptions noted under accomplishments.

4. Impact

Androgen deprivation therapy (ADT) induced by surgical or chemical castration limits the growth of androgen-responsive tissues, but is not curative and ultimately castration-resistant PC results. An innovative therapeutic niche that has not been successfully exploited to date is the persistent population of PC cells after ADT treatment that likely contributes to castration-resistant PC. We and others have demonstrated that a substantial portion of these persistent cells express markers of cellular senescence, a terminal growth arrest characterized by exit from the cell cycle. Persistent senescent cells exhibit a senescent-associated secretory phenotype, which results in enhanced protein translation and the accumulation of misfolded proteins, thereby activating a conserved proteotoxic stress response (PSR) characterized by induction of multiple heat shock protein (Hsp) families coordinated by the master transcriptional regulator Hsf1 that enables cell survival. We propose that this intrinsic PSR may represent a potential 'Achilles heel' that may be exploited to drive persistent PC cells into apoptosis using a synthetic lethal approach. Intriguingly, the safe and widely used diabetes oral agent metformin was recently reported to inhibit the Hsf1-mediated PSR. **The induction of cell senescence using ADT, followed by a synthetic lethal therapy approach using metformin to drive these senescent cells into apoptosis by disrupting the PSR, is a transformative concept has not been addressed to date**. Although components of the PSR such as Hsp27 have been targeted previously, we postulate that our approach will be more effective because it targets the global PSR network, not just one of its many downstream mediators. Notably, metformin is an inexpensive drug with documented safety, even in nondiabetic patients that ***we propose to repurpose*** as synthetic lethal cancer therapy that can be readily translated into the clinic. This proposal addresses the PCRP overarching challenge of mechanisms of resistance in men with high-risk PC.

5. Changes Problems

We are using a modified approach to identify senescent prostate cancer cells after treatment as noted under Major Task 1, Subtask 1.

6. Products

Abstracts:

Metformin Use Associated with Improved Survival in Veterans with Advanced Prostate Cancer: A Large Observational Study. Kyle A. Richards, Jinn-ing Liou, Vincent Cryns, Tracy M. Downs, E. Jason Abel, and David F. Jarrard (American Urological Association Meeting. Boston MA 2017)

Publications:

Strekalova E, Malin D, Rajanala H, **Cryns VL**. Metformin sensitizes triple-negative breast cancer to proapoptotic TRAIL receptor agonists by suppressing XIAP expression. *Breast Cancer Res Treat* 2017;163:435-447.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Senior key personnel have been working on the project since the initiation of the project with no changes.

The following individuals have worked on the project:

Name: David F. Jarrard, MD

Project Role: Principal Investigator

Researcher Identifier (e.g., ORCID ID): 0000-0001-8444-7165

Nearest person month worked: 1.2

Contribution to Project: David Jarrard has conceived and designed the study, reviewed all of the data and the analysis of all of the results on the project, wrote and revised the manuscript.

Name: Vince Cryns, MD

Project Role: Co-Principal Investigator

Researcher Identifier (e.g., ORCID ID): 0000-0003-0355-2268

Nearest person month worked: 2

Contribution to Project: Vince Cryns has conceived and designed the study, reviewed all of the data and the analysis of all of the results on the project, wrote and revised the manuscript.

Name: Kyle Richards, MD

Project Role: Co- Investigator

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 0.3

Contribution to Project: Dr Richards has reviewed the data and the analysis of all of Aim 3 on the project, co-wrote and revised the manuscript.

Name: Shiva DAMODARAN

Project Role: Post-Doctoral Fellow

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 4

Contribution to Project: Shiva has optimized the animal xenografts and metformin treatment and is currently performing the animal studies.

Name: Bing Yang, MD, PhD

Project Role: Researcher

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 3.6

Contribution to Project: Bing Yang has prepared all the PCa cell lines used in this study and performed the analysis on the cell lines, organized the data and assisting with the mouse studies.

Name: Joe Gawdzik, PhD

Project Role: Post-Doctoral Fellow

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 1

Contribution to Project: Joe has assisted with the cell culture experiments for Task 1 and data analysis and collection.

Name: Jinn-ing Liou

Project Role: Researcher

Researcher Identifier (e.g., ORCID ID):

Nearest person month worked: 0.3

Contribution to Project: Jinn-ing has generated the data and the analysis of all of Aim 3 on the project, co-wrote and revised the manuscript.

Name: Dmitry Malin, PhD

Project Role: Associate Scientist

Researcher Identifier (e.g., ORCID ID): 0000-0002-5728-7511

Nearest person month worked: 6

Contribution to Project: Dmitry Malin has analyzed human PCa cell lines for markers of proteotoxic stress in response to androgen deprivation therapy with or without metformin treatment and assisted with the design of these experiments.

8. APPENDICES:

Abstract and manuscript.

Metformin Use Associated with Improved Survival in Veterans with Advanced Prostate Cancer: A Large Observational Study

Kyle A. Richards, Jinn-ing Liou, Vincent Cryns, Tracy M. Downs, E. Jason Abel, and David F. Jarrard

Size: The size of the abstract is limited to 2,280 characters not including spaces.

This includes title, body of abstract, tables and graphics. Tables are calculated at 225 characters per table. Graphics are calculated at 225 characters per graphic.

Background: Metformin is a commonly prescribed 1st line glucose-lowering therapy for patients with type 2 diabetes mellitus. There is increasing clinical data supporting the anti-neoplastic effects of metformin for various cancers including prostate cancer (PCa). In addition, we hypothesize that metformin plus androgen deprivation therapy (ADT) may be synergistic in men with advanced PCa. Hence the objective of this study was to assess the impact of metformin plus ADT in a retrospective cohort of Veterans with advanced PCa.

Methods: Using national Veterans Affairs databases, we identified all men diagnosed with PCa between 2000-2008 that were treated with ADT with follow-up through October of 2015. We excluded patients that were treated with ADT for ≤6 months or were receiving ADT concurrently with localized radiation therapy. We split these patients into three cohorts: 1. Patients without diabetes 2. Diabetics on metformin 3. Diabetics no metformin. Our primary outcome was overall survival (OS) and secondary outcomes included skeletal related events (SRE) and prostate-cancer specific survival. Cox proportional hazards ratios were calculated for overall and disease specific survival.

Results: The total cohort after exclusions consisted of 87,344 patients of which 53,893 (61%) were non-diabetics, 14,517 (17%) were diabetics on metformin, and 18,934 (22%) were diabetics not receiving metformin. The mean age was 75 ±11 years in the non-diabetics, 71 ±12 in the diabetics on metformin, and 75 ±10 in the diabetics no metformin ($p<0.001$). The median OS was 7.1 years in the non-diabetics, 9.1 years in the diabetics on metformin, and 7.4 years in the diabetics no metformin.

Multivariable Cox proportional hazards analysis assessing for predictors of overall survival showed improved survival in diabetics on metformin (HR 0.77, 95% CI 0.74-0.81) vs. diabetics no metformin (HR 0.99, 95% CI 0.95-1.03) with non-diabetics as referent group. Multivariable Cox proportional hazards analysis assessing for predictors of SRE revealed no association between metformin use (HR 0.99, 95% CI 0.92-1.07) and SRE. Lastly, multivariable Cox proportional hazards analysis assessing for predictors of prostate-cancer specific survival showed improved survival in diabetics on metformin (HR 0.72, 95% CI 0.67-0.78) and to a lesser effect diabetics no metformin (HR 0.87, 95% CI 0.81- 0.93) with non-diabetics as referent group.

Conclusion: Metformin use in Veterans with advanced prostate cancer receiving ADT is associated with improved OS and cancer-specific survival. The impact of metformin in prostate cancer patients should be evaluated in a prospective clinical trial.

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PRECLINICAL STUDY

Metformin sensitizes triple-negative breast cancer to proapoptotic TRAIL receptor agonists by suppressing XIAP expression

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Abstract
Purpose Despite robust antitumor activity in diverse preclinical models, TNF-related apoptosis-inducing ligand (TRAIL) receptor agonists have not demonstrated efficacy in clinical trials, underscoring the need to identify agents that enhance their activity. We postulated that the metabolic stress induced by the diabetes drug metformin would sensitize breast cancer cells to TRAIL receptor agonists.
Methods Human triple (estrogen receptor, progesterone receptor, and HER2)-negative breast cancer (TNBC) cell lines were treated with TRAIL receptor agonists (monoclonal antibodies or TRAIL peptide), metformin, or the combination. The effects on cell survival, caspase activation, and expression of TRAIL receptors and the antiapoptotic protein XIAP were determined. In addition, XIAP was silenced by RNAi in TNBC cells and the effects on sensitivity to TRAIL were determined. The antitumor effects of metformin, TRAIL, or the combination were evaluated in an orthotopic model of metastatic TNBC.
Results Metformin sensitized diverse TNBC cells to TRAIL receptor agonists. Metformin selectively enhanced the sensitivity of transformed breast epithelial cells to TRAIL receptor agonist-induced caspase activation and apoptosis with little effect on untransformed breast epithelial cells. These effects of metformin were accompanied by robust reductions in the protein levels of XIAP, a negative regulator of TRAIL-induced apoptosis. Silencing XIAP in TNBC cells mimicked the TRAIL-sensitizing effects of metformin. Metformin also enhanced the antitumor effects of TRAIL in a metastatic murine TNBC model.
Conclusions Our findings indicate that metformin enhances the activity of TRAIL receptor agonists, thereby supporting the rationale for additional translational studies combining these agents.

Keywords Metformin · Breast cancer · TRAIL · Metastasis · Apoptosis · Therapeutics

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) and agonistic mAbs targeting its proapoptotic receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) selectively activate the caspase-8/10-mediated extrinsic apoptotic pathway in transformed cells and exhibit robust antitumor effects in diverse murine models of cancer [1, 2]. Despite promising preclinical results, TRAIL receptor agonists have failed to demonstrate significant efficacy, either alone or in combination with chemotherapy, in multiple clinical trials in advanced malignancies [3–8]. These disappointing results in clinical trials have been attributed to a number of factors, including de novo and/or acquired resistance to TRAIL receptor agonists, inadequate receptor oligomerization on ligand binding, limiting procaspase-8/10 activation, and lack of biomarkers to predict treatment response [1, 2].

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In an effort to enhance the activity of TRAIL receptor agonists, these proapoptotic agents have been combined with TRAIL-sensitizing agents (e.g., histone deacetylase inhibitors, PPARγ agonists, aspirin, mTOR, and other kinase inhibitors) to augment its antitumor effects [9–13]. More recently, we have identified a novel nutritional

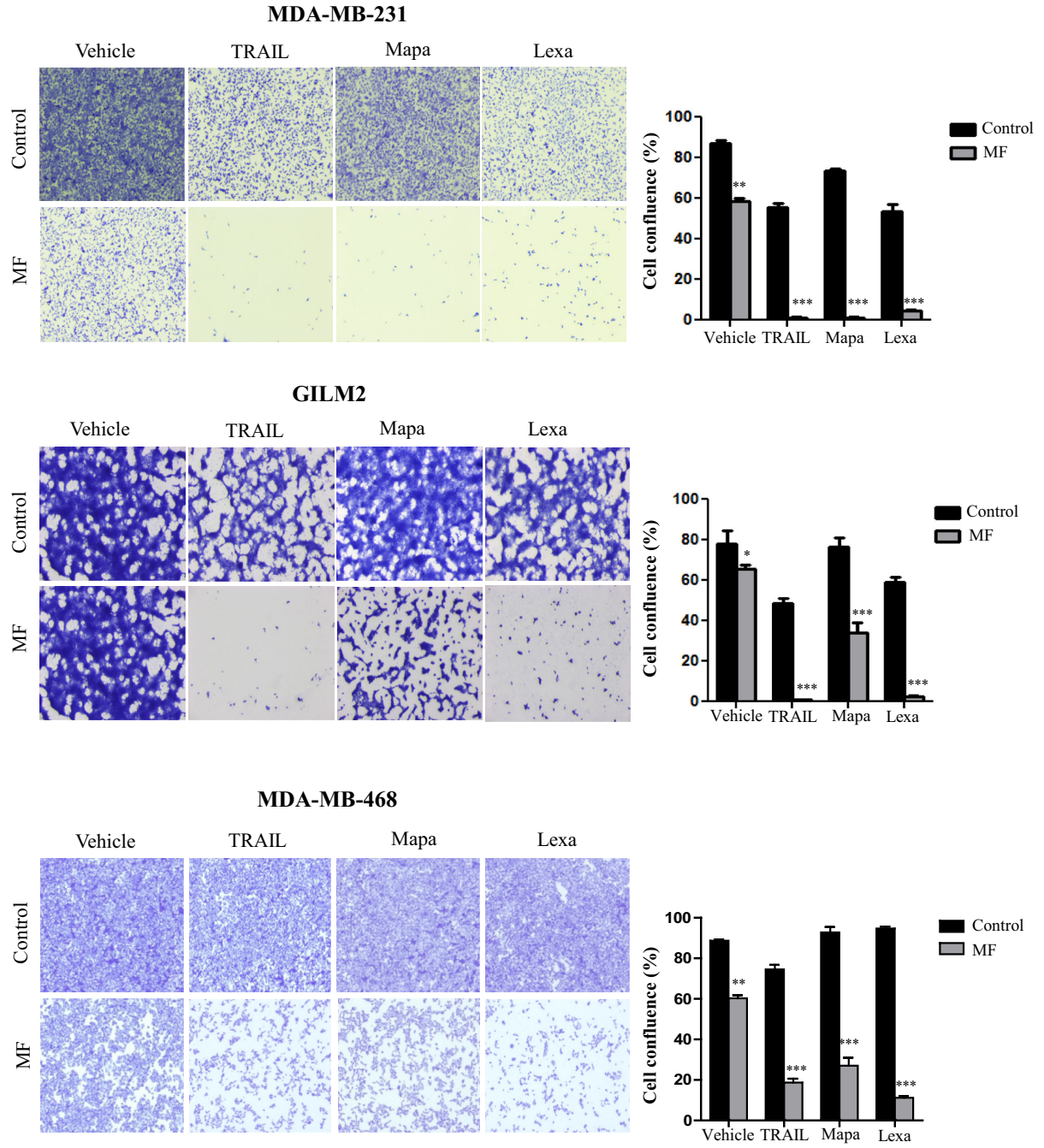


Fig. 1 Metformin sensitizes TNBC cells to TRAIL receptor agonists. Crystal violet cell survival assay of TNBC cells preincubated with vehicle or metformin (1 mM for MDA-MB-468 cells or 5 mM for MDA-MB-231-mCherry and GILM2 cells) for 48 h, and treated with vehicle or TRAIL, mapatumumab, or lexatumumab (each 1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for an additional 24 h (MDA-MB-231-mCherry and GILM2 cells) or 48 h (MDA-MB-468 cells). *Left panels* representative images. *Right panels* the percentage confluence of crystal violet-positive cells was scored (mean ± SEM, $n = 3$). In all panels, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

intervention that selectively sensitizes breast cancer cells to TRAIL-R2 agonists [14]. Specifically, we demonstrated that depletion of the essential amino acid methionine metabolically primes breast cancer cells to respond to the agonistic TRAIL-R2 mAb lexatumumab by increasing cell surface expression of TRAIL-R2. Moreover, dietary methionine restriction enhanced the antitumor activity of lexatumumab against mammary tumors and lung metastases in an orthotopic model of metastatic breast cancer [14]. Hence, methionine restriction metabolically primes breast cancer cells to targeted agents that activate cell death by exposing a targetable vulnerability, namely enhanced cell surface expression of TRAIL-R2.

Intriguingly, the diabetes medication metformin mimics many of the effects of methionine restriction, including disruption of methionine metabolism via inhibition of the functionally linked folate cycle in the one-carbon metabolic pathway, inhibition of the mechanistic target of rapamycin (mTOR), broad antitumor activity, improved insulin sensitivity, and prolonged lifespan [15–19]. Metformin use has been associated with reduced incidence of a broad range of tumor types and reduced cancer mortality in many epidemiologic studies [19–22]. However, the antitumor mechanisms of metformin are not well understood. Both direct tumor effects (activation of AMPK with resultant inactivation of mTORC1, inhibition of mitochondrial complex I, and suppression of nuclear translocation of NFκB and Stat3 phosphorylation) and indirect systemic effects (reduction in insulin and IGF-1 levels) have been reported and implicated in its antitumor effects [18, 19]. Consistent with preclinical findings, metformin treatment of newly diagnosed breast cancer patients prior to surgery improves metabolic indices, increases tumor apoptosis, and decreases tumor proliferation [23].

Given the similarities noted between methionine restriction and metformin, we postulated that metformin may also metabolically prime breast cancer cells to respond to proapoptotic TRAIL receptor agonists. Here we report that metformin sensitizes TNBC cells to TRAIL receptor agonists in vitro and in vivo. Metformin selectively

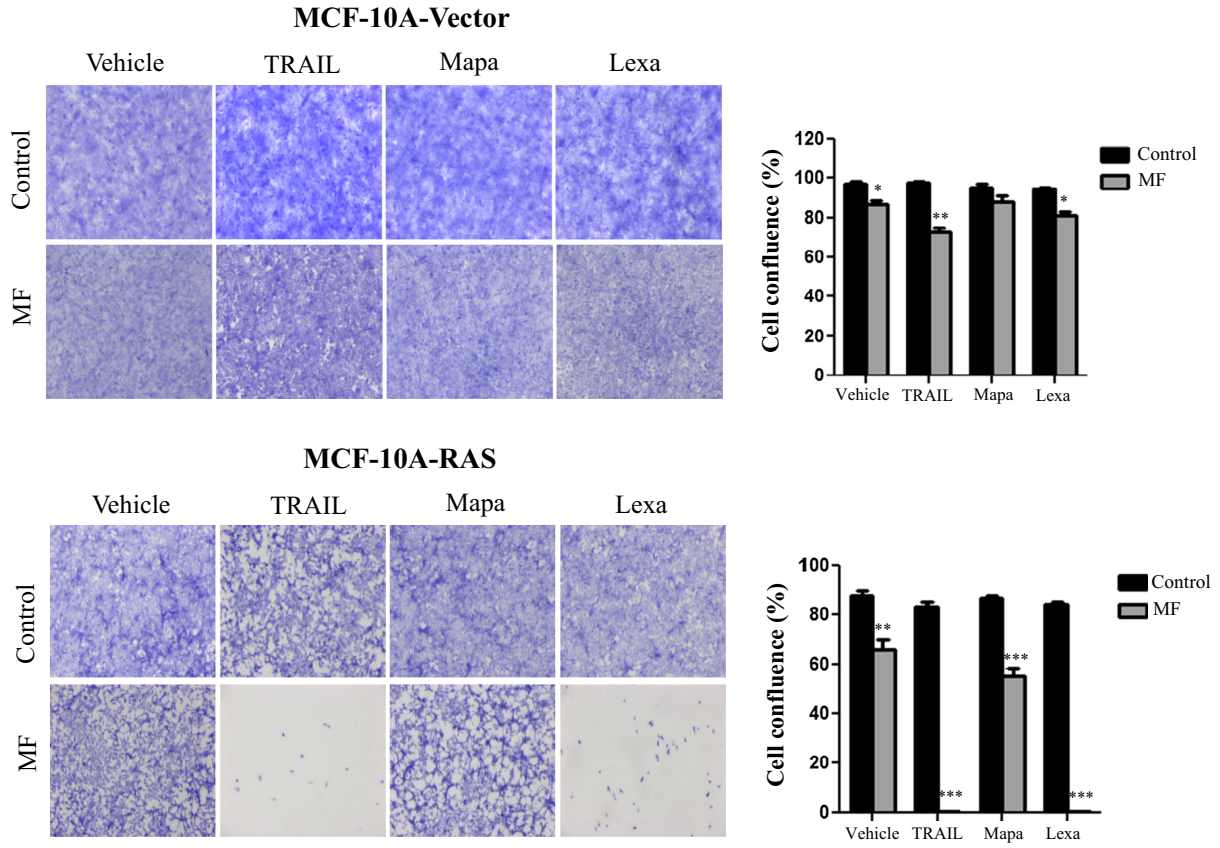


Fig. 2 Metformin sensitizes transformed breast epithelial cells to TRAIL receptor agonists. Crystal violet cell survival assay of MCF-10A-Vector or MCF-10A-Ras cells preincubated with vehicle or metformin (5 mM) for 48 h, and then treated with vehicle or TRAIL, mapatumumab, or lexatumumab (each 1.5 μg/ml) for an additional 24 h. *Left panels* representative images. *Right panels* the percentage confluence of crystal violet-positive cells was scored (mean ± SEM, $n = 3$). In all panels, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

enhances the sensitivity of transformed breast epithelial cells to TRAIL receptor agonist-induced caspase activation and apoptosis with little effect on untransformed breast epithelial cells. These effects of metformin are accompanied by a robust reduction in the expression level of the antiapoptotic protein XIAP. Metformin also enhances the antitumor effects of TRAIL in a murine model of TNBC. Collectively, our findings indicate that metformin enhances the clinical activity of TRAIL receptor agonists, and suggests that additional translational studies combining these agents are warranted.

Methods and materials

Cell culture and reagents

Human MDA-MB-231 and GILM2 TNBC cells stably expressing mCherry and parental GILM2 TNBC cells were maintained as described [24]. MDA-MB-468 TNBC cells were cultured in DMEM media supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin (ThermoFisher Scientific/Gibco). Human MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or vector were maintained as described [25]. Mapatumumab and lexatumumab were kindly provided by Dr. Robin Humphreys (Human Genome Sciences). Recombinant TRAIL peptide (amino acids 95–281) was purified as described [11].

Crystal violet cell survival assay

A crystal violet cell survival assay was performed as described (10). Cells were seeded on 6-well plates (3×10^5 cells/well) overnight. Cells were then preincubated with vehicle or metformin (1 mM for MDA-MB-468 cells or 5 mM for MDA-MB-231-mCherry and GILM2 cells) for 48 h, and treated with vehicle or TRAIL, mapatumumab, or lexatumumab (each 1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for an additional 24 h (MDA-MB-231-mCherry and GILM2 cells) or 48 h (MDA-MB-468 cells) before staining with crystal violet. The percentage cell confluence of crystal violet-positive cells was determined in 3 fields per treatment condition using NIH ImageJ software. Cells in each field were colored using "color threshold." Percentage confluence of colored cells was quantified using "analyze particles" which reports the percent area in each field occupied by colored cells.

Immunoblotting

Whole-cell lysates were immunoblotted as described [25] using primary Abs against XIAP, β-actin (Sigma-Aldrich), PARP (BD Biosciences), and procaspase-3 (Cell Signaling Technology).

Fig. 3 Metformin enhances TRAIL-induced caspase activation in TNBC cells. **a** Immunoblots of TNBC cells preincubated with vehicle or metformin (5 mM) for 48 h and then treated with vehicle or TRAIL (1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for 16 h. **b** TNBC cells were preincubated with vehicle or metformin (5 mM) for 48 h, treated with vehicle or TRAIL (1.5 μg/ml for MDA-MB-231-mCherry and GILM2 cells or 4 μg/ml for MDA-MB-468 cells) for 24 h, and caspase-3/7 activity was measured (mean ± SEM, n = 3). **c** Immunoblots of MCF-10A breast epithelial cells stably expressing vector or H-RasV12 preincubated with vehicle or metformin (5 mM) for 48 h and then treated with vehicle or TRAIL (1.5 μg/ml) for 16 h. **d** MCF-10A-Vector or MCF-10A-RAS cells were preincubated with vehicle or metformin (5 mM) for 48 h, treated with vehicle or TRAIL (1.5 μg/ml) for 24 h, and caspase-3/7 activity was measured (mean ± SEM, $n = 3$). In **b**, **d** $^{**}P < 0.01$ and $^{***}P < 0.001$

Caspase-3/7 activity assay

The Caspase-Glo 3/7 Assay System (Promega) was used to measure caspase-3/7 activity in cell lysates according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates (2.5×10^3 cells/well) overnight. The next day, cells were preincubated with vehicle or metformin (5 mM) for 48 h and then treated with vehicle or TRAIL (1.5 μg/ml for MDA-MB-231-mCherry, GILM2, MCF-10A and MCF-10A-RAS cells, or 4 μg/ml for MDA-MB-468 cells) for an additional 24 h. Caspase-3/7 activity was normalized to cell number and expressed as fold activity compared to vehicle-treated cells.

TRAIL receptor cell surface expression

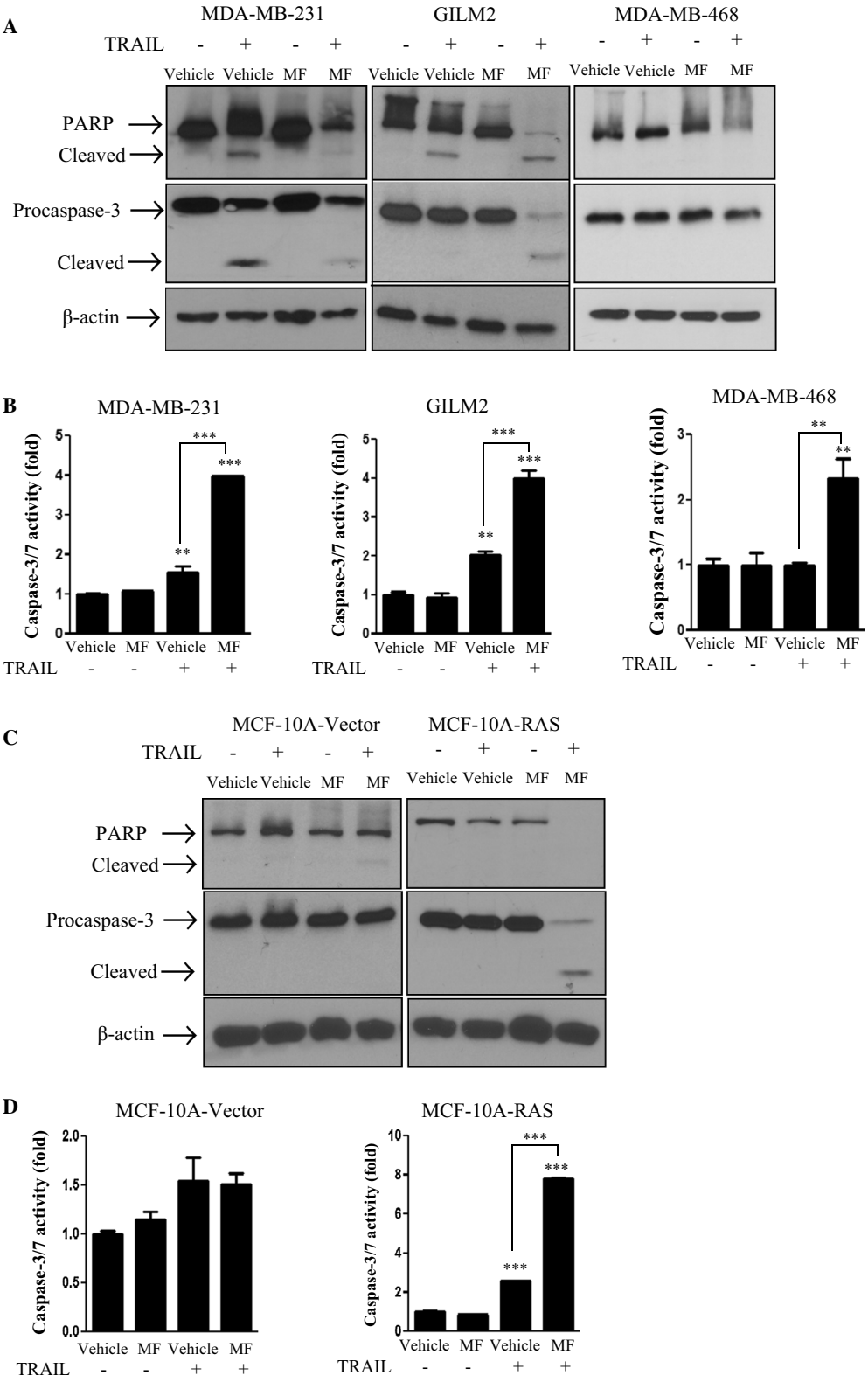
Cell surface expression of TRAIL receptors (TRAIL-R1 and TRAIL-R2) was determined by flow cytometry using TRAIL-R1, TRAIL-R2, or control IgG1 mAb conjugated with allophycocyanin (BioLegend) as described previously [14].

XIAP siRNA experiments

siRNAs targeting the sequences GAAGCUAGAUUAAAG UCCU (si1-XIAP) or CAGUGAAGACCCUUGGGAA (si2-XIAP) in human XIAP and non-silencing control siRNA were purchased from Sigma-Aldrich. Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (ThermoFisher Scientific) according to the manufacturer's protocol.

Real-time PCR

Real-time PCR for TRAIL-R1, TRAIL-R2, and GAPDH was performed as described previously [14]. Primers for XIAP (forward 5-AGTGCCACGCAGTCTACAAA,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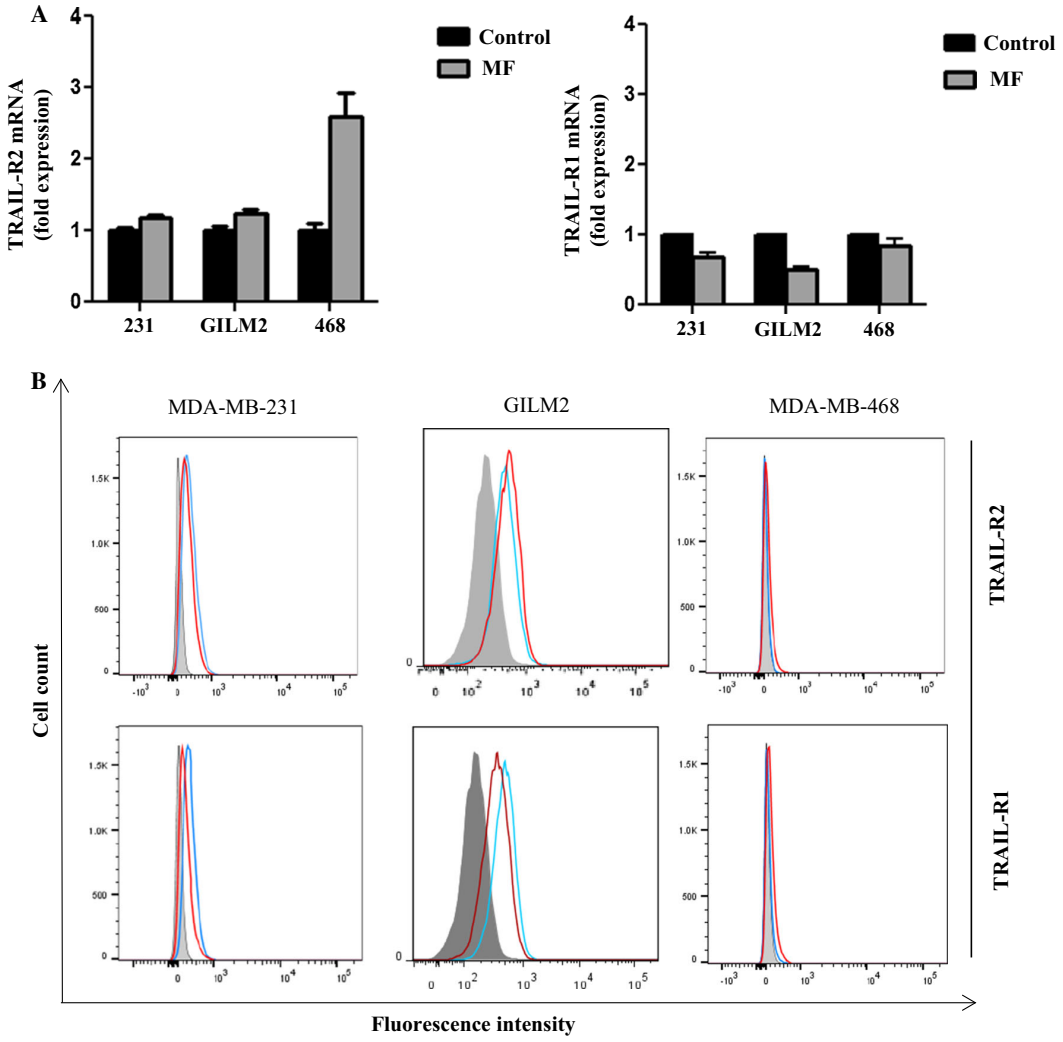


Fig. 4 Metformin does not alter cell surface expression of TRAIL receptors in TNBC cells. **a** TNBC cells were treated with vehicle or metformin (5 mM) for 72 h. TRAIL-R2 (*left panel*) and TRAIL-R1 (*right panel*) mRNA levels were determined by real-time PCR and were normalized to expression in vehicle-treated TNBC cells. **b** TNBC cells were treated with vehicle or MF (5 mM) for 72 h and then incubated with IgG, TRAIL-R1, or TRAIL-R2 Ab. Cell surface expression of TRAIL receptors was determined by flow cytometry. *Gray bar* TNBC cells incubated with IgG. *Blue line* TNBC cells treated with vehicle and incubated with TRAIL-R1 (*bottom panels*) or TRAIL-R2 (*top panels*) Ab. *Red line* TNBC cells treated with metformin and incubated with TRAIL-R1 (*bottom panels*) or TRAIL-R2 (*top panels*) Ab

reverse 5-GCATGTGTCTCAGATGGCCT) were purchased from Integrated DNA Technologies and real-time PCR was performed using the same methods. A comparative Ct method was used to normalize RNA expression in samples to the controls in each experiment.

Orthotopic model of metastatic TNBC

GILM2-mCherry TNBC cells (2×10^6) were resuspended in Matrigel (BD Biosciences) and injected bilaterally into the 4th mammary gland ducts of 4- to 5-week-old female NOD *scid* IL2 receptor γ chain knockout (NSG) mice (Jackson Laboratory). Mice were randomized into four treatment groups (10 mice per group) three weeks after tumor inoculation: (1) PBS vehicle *i.p.* daily; (2) metformin 2 mg/ml in the drinking water; (3) TRAIL (10 mg/kg *i.p.* daily), or (4) metformin (2 mg/ml in the drinking water) plus TRAIL (10 mg/kg *i.p.* daily). Mammary tumor volume was calculated as described [26]. Lung metastases were visualized by fluorescence microscopy in isolated whole lungs and scored using NIH ImageJ analysis as described [26]. All animal experiments were carried out as

part of an IACUC-approved protocol at the University of Wisconsin–Madison.

Tumor apoptosis assay

Formalin-fixed, paraffin-embedded tumor tissue sections were analyzed for active caspase-3 expression by immunohistochemistry using an Ab against cleaved caspase-3 (Cell Signaling) as described [26].

Statistics

The statistical significance of differences between groups was determined by ANOVA with Bonferroni post tests using GraphPad Prism 4 software.

Results

Metformin sensitizes TNBC cells to TRAIL receptor agonists

To determine whether metformin sensitizes TNBC to TRAIL receptor agonists, three human TNBC cell lines (MDA-MB-231-mCherry, GILM2, and MDA-MB-468) were preincubated with vehicle or metformin and then treated with TRAIL, mapatumumab, or lexatumumab. Metformin sensitized all three TNBC cell lines to TRAIL receptor agonists, with the most robust effects observed in MDA-MB-231-mCherry and GILM2 cells. (Figure 1). A dose–response experiment in MDA-MB-468 cells identified 0.5 mM metformin as the minimal concentration needed to sensitize these TNBC cells to TRAIL receptor agonists (Fig. S1). In addition, preincubation with metformin sensitized human HT29 colon adenocarcinoma and DU145 prostate cancer cell lines to TRAIL receptor agonists (Fig. S2). Collectively, these results demonstrate that metformin augments the cytotoxicity of TRAIL receptor agonists against a broad range of tumor cell types, including TNBC cells.

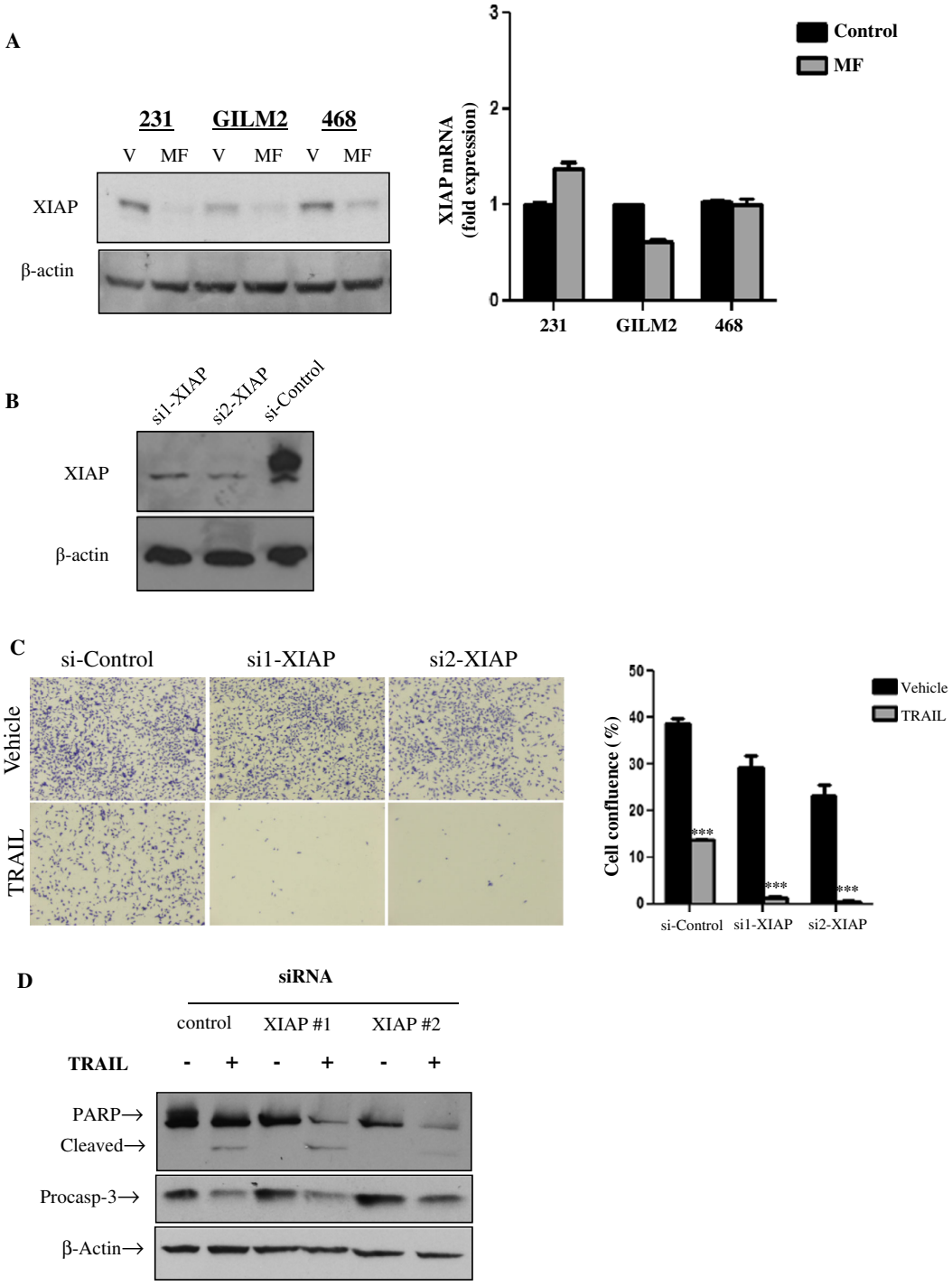
Metformin sensitizes transformed breast epithelial cells to TRAIL receptor agonists

To determine whether metformin enhanced the cytotoxicity of TRAIL receptor agonists preferentially in transformed cells, we utilized human MCF-10A breast epithelial cells transformed by oncogenic H-RasV12 and untransformed MCF-10A cells stably expressing empty vector. Strikingly, metformin robustly sensitized MCF-10A-Ras cells to TRAIL and lexatumumab, but had a minimal effect on the sensitivity of untransformed MCF-10A-Vector cells to these agents (Fig. 2). In contrast, metformin had only a modest effect on the cytotoxicity of mapatumumab against MCF-10A-Ras cells. Collectively, these results indicate that metformin preferentially sensitizes transformed cells to TRAIL receptor agonists and support the potential tumor selectivity of this therapeutic combination.

Fig. 5 Metformin reduces XIAP protein expression in TNBC cells and silencing XIAP sensitizes TNBC cells to TRAIL. **a** Immunoblot of XIAP protein levels (*left panel*) and real-time PCR analysis of XIAP mRNA levels (*right panel*) in TNBC cells treated with vehicle or metformin (5 mM) for 72 h (immunoblots) or 48 h (real-time PCR). **b** MDA-MB-231-mCherry cells were transfected with a non-silencing control siRNA (si-Control) or one of two different siRNAs targeting XIAP (si1-XIAP or si2-XIAP). Immunoblot of XIAP expression 48 h after transfection. **c** Crystal violet cell survival assay of MDA-MB-231-mCherry cells transfected with control or XIAP siRNAs, and 48 h later treated with vehicle or TRAIL (0.3 μg/ml) for 24 h. *Left panel* representative images. *Right panel* quantification of cell confluence in 3 fields for each treatment (mean ± SEM, $n = 3$) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **d**, Silencing XIAP enhances TRAIL-induced caspase activation in TNBC cells. MDA-MB-231-mCherry cells were transfected with control or XIAP siRNAs, and 48 h later were treated with vehicle or TRAIL (0.3 μg/ml) for 16 h. PARP (full-length and caspase-cleaved) and procaspase-3 levels were determined by immunoblotting

Metformin enhances TRAIL-induced caspase activation in TNBC cells

To investigate whether metformin promotes TRAIL-induced caspase activation, TNBC cells were preincubated with metformin, treated with vehicle or TRAIL, and then analyzed by immunoblotting. Metformin promoted TRAIL-induced proteolysis of the caspase substrate PARP as detected by diminished full-length PARP and/or increased cleavage product compared to vehicle-treated cells (Fig. 3a). In addition, metformin enhanced TRAIL-induced procaspase-3 proteolytic processing as detected by decreased procaspase-3 levels and/or increased cleaved subunit compared to vehicle-treated cells. Similarly, pretreatment of HT29 and DU145 carcinoma cells with metformin augmented TRAIL-induced caspase activation (Fig. S3). To quantitate more precisely the effects of metformin and TRAIL on caspase activation, we utilized a caspase-3/-7 activity assay. Metformin robustly enhanced TRAIL-induced caspase-3/-7 activity in all three TNBC cells compared to cells treated with either agent alone (Fig. 3b). Metformin also enhanced TRAIL-induced caspase activation in MCF-10A-Ras cells, while untransformed MCF-10-Vector cells were not sensitive to this combination (Fig. 3c, d). Consistent with its reported mechanism of action [18, 19], metformin increased p-AMPK levels and selectively inhibited mTORC1 as determined by reduced phosphorylation of the mTORC1


A
231
GILM2
468
V
MF
V
MF
V
MF
XIAP
β-actin
XIAP mRNA (fold expression)
Control
MF
231
GILM2
468
B
si1-XIAP
si2-XIAP
si-Control
XIAP
β-actin
C
si-Control
si1-XIAP
si2-XIAP
Vehicle
TRAIL
Cell confluence (%)
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TRAIL
***
si-Control
si1-XIAP
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D
siRNA
control
XIAP #1
XIAP #2
TRAIL
PARP→
Cleaved→
Procasp-3→
β-Actin→

substrate p-S6 and no effect on the mTORC2 substrate p-Akt (Fig. S4). Taken together, these findings indicate that metformin potently augments caspase activation and apoptosis by TRAIL in a broad range of tumor cell types and provide additional evidence for the tumor selectivity of this combination.

Metformin does not alter cell surface expression of TRAIL receptors in TNBC cells

To determine whether metformin sensitizes cancer cells to TRAIL by increasing the expression of its proapoptotic receptors (TRAIL-R1 and TRAIL-R2), TNBC cells were treated with metformin and TRAIL receptor mRNA levels were measured by real-time PCR. Metformin treatment resulted in a modest increase in TRAIL-R2 mRNA levels in MDA-MB-468 TNBC cells, but had little effect on TRAIL-R2 levels in the other TNBC cell lines or on TRAIL-R1 mRNA levels in any of the TNBC cell lines (Fig. 4a). Furthermore, metformin did not significantly affect cell surface expression of either proapoptotic TRAIL-R1 or TRAIL-R2 receptors as determined by flow cytometry (Fig. 4b). Metformin also did not alter protein levels of MAGED2 (Fig. S5), which we previously demonstrated to be downregulated by methionine restriction [14]. These findings indicate that the TRAIL-sensitizing effects of metformin are not due to enhanced TRAIL receptor expression or cell surface localization in TNBC cells.

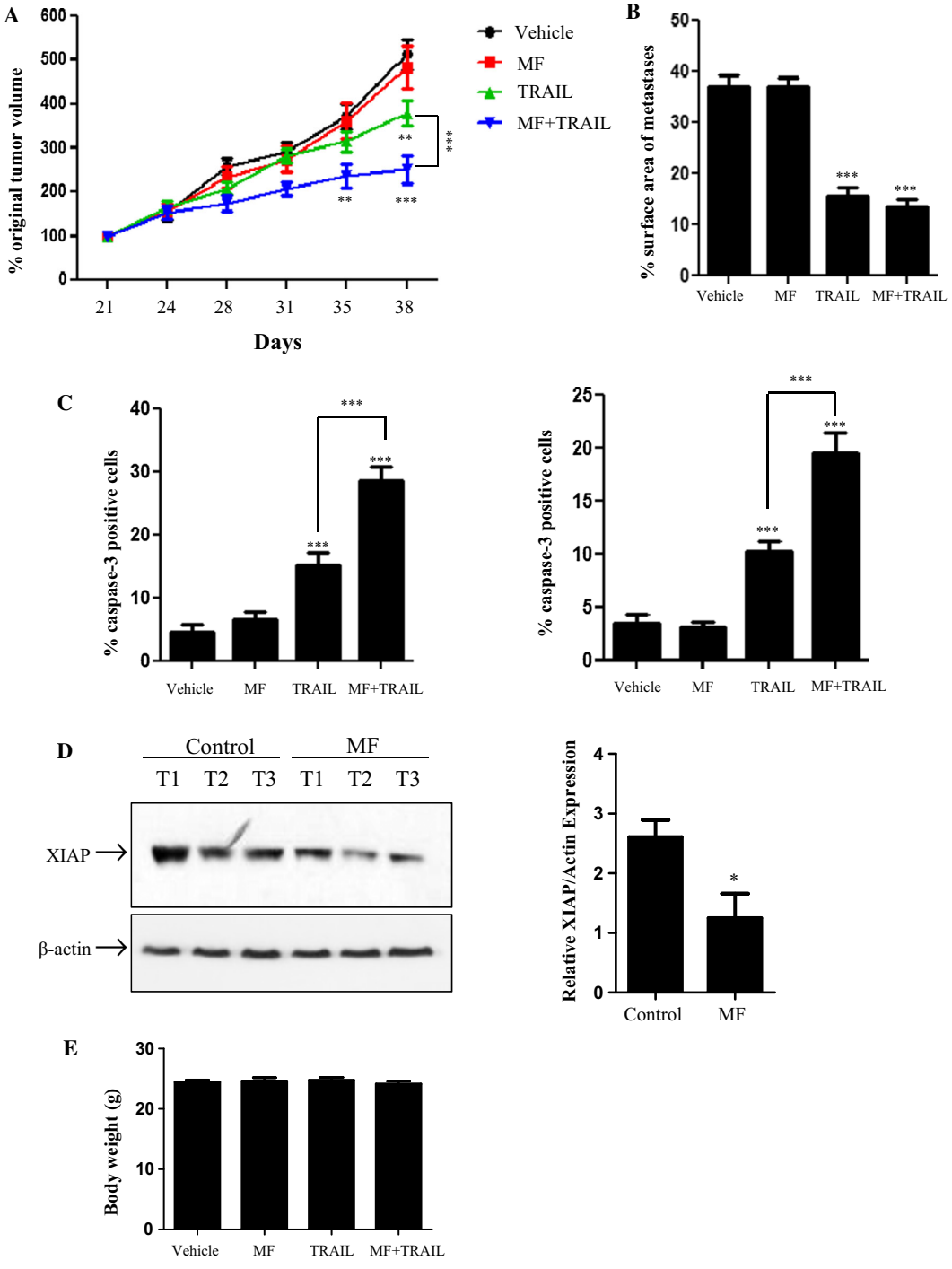
Metformin reduces XIAP expression in TNBC cells and silencing XIAP sensitizes TNBC cells to TRAIL

The antiapoptotic X-linked inhibitor of apoptosis protein (XIAP) has been demonstrated to confer resistance to TRAIL-induced apoptosis by suppressing caspase activation [27, 28]. Therefore, we postulated that metformin might sensitize cancer cells to TRAIL by downregulating XIAP. Consistent with this hypothesis, treatment of MDA-MB-231-mCherry, GILM2, and MDA-MB-468 TNBC cells with metformin resulted in a reduction in XIAP protein levels (Fig. 5a, left panel). In contrast, treatment with metformin did not affect XIAP mRNA levels (Fig. 5a, right panel), indicating that metformin regulates XIAP via a posttranscriptional mechanism. To examine the functional role of XIAP downregulation in the TRAIL-sensitizing effects of metformin, MDA-MB-231 cells were transfected with a scrambled siRNA (si-Control) or one of two different siRNAs targeting XIAP (si-1 XIAP and si-2 XIAP). Both siRNAs targeting XIAP reduced XIAP protein levels compared to the scrambled control siRNA (Fig. 5b). Notably, silencing XIAP in MDA-MB-231-mCherry cells had a modest effect on cell viability and robustly sensitized these cells to TRAIL treatment compared to a scrambled control siRNA (Fig. 5c). To examine whether silencing XIAP enhanced TRAIL-induced caspase activation, MDA-MB-231-mCherry cells were transfected with siRNAs targeting XIAP or a scrambled control and then treated overnight with vehicle or TRAIL. Silencing XIAP enhanced TRAIL-induced cleavage of the caspase substrate PARP (reduction of full-length PARP and/or increased cleavage product), while levels of procaspase-3 proteolysis (reduction of procaspase-3 levels) were comparable in TRAIL-treated cells transfected with scrambled siRNAs or siRNAs targeting XIAP (Fig. 5d). Collectively, these results indicate that metformin sensitizes TNBC cells to TRAIL-induced apoptosis by downregulating expression of XIAP.

Fig. 6 Metformin enhances the antitumor effects of TRAIL in an orthotopic model of metastatic TNBC. Three weeks after intraductal injection of tumor cells, female NSG mice with GILM2-mCherry mammary tumors were randomized into 4 groups (10 mice per group): control group (PBS *i.p.* daily for three weeks), metformin (2 mg per ml in drinking water for three weeks), TRAIL (10 mg/kg *i.p.,* daily for three weeks), or metformin plus TRAIL (same dosing as in single-agent arms). **a** The percentage of the original mammary tumor volume (at 3 weeks) in each group (mean ± SEM, $n = 10$ mice per group). **b** The percentage of the surface area occupied by lung metastases (mean ± SEM, $n = 10$ mice per group). **c** The percentage active caspase-3-positive tumor cells in mammary tumors (*left panel*) or metastatic lung tumors (*right panel*) after treatment (mean ± SEM, $n = 3$ tumors per group). **d** Immunoblot of XIAP expression in mammary tumors from control vehicle-treated or metformin-treated mice ($n = 3$ mammary tumors per group). XIAP expression normalized to β-actin was measured by Image J analysis. **e** Body weight of the mice in each treatment group at the end of the study (mean ± SEM, $n = 10$ mice per group). In all panels, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated mice or the indicated comparison

Metformin enhances the antitumor effects of TRAIL in an orthotopic model of metastatic TNBC

To determine whether metformin augments the antitumor effects of TRAIL in an orthotopic model of metastatic TNBC, we treated female NSG mice bearing established GILM2-mCherry mammary tumors with vehicle, metformin alone, TRAIL alone, or the combination of metformin and TRAIL. Under the conditions tested, metformin had no significant effect on mammary tumor growth or lung metastases. In contrast, TRAIL inhibited mammary tumor growth, but the combination of TRAIL and metformin was more effective than TRAIL alone (Fig. 6a). Both therapies, TRAIL alone or in combination with metformin, inhibited lung metastases to a comparable degree (Fig. 6b). Moreover, both TRAIL alone or in combination with metformin induced apoptosis in mammary tumors and lung metastatic lesions as determined by


A
% original tumor volume
Days
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TRAIL
MF+TRAIL
B
% surface area of metastases
C
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XIAP
β-actin
Relative XIAP/Actin Expression
E
Body weight (g)

active, cleaved caspase-3 immunostaining (Fig. 6c). Consistent with our in vitro findings, the combination of TRAIL and metformin resulted in more apoptosis induction in mammary tumors and lung metastases than either TRAIL or metformin alone. Importantly, metformin treatment reduced XIAP protein levels in mammary tumors (Fig. 6d), consistent with our in vitro findings. None of the treatments affected the body weight of the mice at the end of the study compared to control vehicle-treated mice (Fig. 6e). Collectively, these findings indicate that metformin enhances the antitumor activity of TRAIL in vivo and provide preclinical evidence supporting additional translational studies investigating the combination of metformin and TRAIL receptor agonists in metastatic TNBC.

Discussion

Based largely on epidemiologic data and promising preclinical studies, the oral diabetes medication metformin has been incorporated into a multitude of clinical trials in diverse early-stage and advanced malignancies, either as monotherapy or in combination with cytotoxic agents or radiation [19]. We have demonstrated that metformin sensitizes TNBC cells to proapoptotic TRAIL receptor agonists, while untransformed breast epithelial cells are largely resistant to this combination. Although other groups have recently demonstrated that metformin enhances TRAIL-induced apoptosis in cultured cancer cells [29–32], we have provided the first in vivo evidence for the therapeutic utility of this combination in an orthotopic model of metastatic TNBC that recapitulates many features of the human disease [24]. Specifically, we showed that metformin augments the antitumor effects of TRAIL against mammary tumors and increases apoptosis in mammary tumors and lung metastases compared to treatment with either agent alone. Importantly, these antitumor effects in vivo were observed at metformin doses that were well tolerated by the mice, a critical point because the TRAIL-sensitizing effects of metformin in vitro typically require millimolar concentrations of metformin in standard cell culture media due to their supraphysiologic glucose concentration [33, 34]. Given the lack of efficacy of TRAIL receptor agonists in clinical trials in advanced solid tumors [3–8], our findings are particularly significant from a translational perspective because they suggest that metformin may enhance the efficacy of TRAIL receptor agonists in clinical trials. As such, our results provide the first murine model evidence for the clinical utility of the combination of metformin and TRAIL receptor agonists, thereby providing critical preclinical evidence to support additional translational studies in poor prognosis of TNBC, which currently lack targeted therapies [35].

Indeed, metformin is a particularly attractive TRAIL-sensitizing agent based on its well-documented safety in both diabetic and non-diabetic patients, its low cost, and its beneficial impact on metabolic health by improving insulin sensitivity [18, 19]. In addition, our results indicate that the combination of metformin and TRAIL receptor agonists activates apoptosis in p53 mutant TNBC cells and preferentially induces apoptosis in transformed breast epithelial cells with little effect on untransformed breast epithelial cells. Although the mechanism of this tumor selectivity remains poorly understood, our findings suggest that the toxicity of the combination therapy may be modest. Moreover, our studies may provide insights into the reduced cancer incidence in individuals treated with metformin reported in many epidemiologic studies [19–22]: metformin may enhance the sensitivity of nascent tumors to TRAIL-dependent immune surveillance pathways.

Mechanistically, we have demonstrated that metformin sensitizes TNBC cells to TRAIL receptor agonists by downregulating the antiapoptotic protein XIAP. XIAP is a direct inhibitor of caspases that confers resistance to TRAIL-induced caspase-3 activation and apoptosis, while induction of TRAIL-induced apoptosis requires XIAP to be displaced from caspases by mitochondrial release of Smac/DIABLO [27, 28]. We observed that metformin reduces protein expression of XIAP but does not alter XIAP mRNA levels, thereby pointing to a posttranslational mechanism. Intriguingly, rapamycin was previously demonstrated to negatively regulate protein translation of XIAP [36], suggesting that metformin may reduce XIAP protein levels via inhibition of mTORC1. Moreover, metformin treatment reduced XIAP protein levels in mammary tumors in our murine model of TNBC, indicating that the dosing used in our study was sufficient to target this pathway in vivo. To determine the functional relevance of the downregulation of XIAP protein by metformin, we used RNAi and observed that XIAP silencing robustly sensitizes TNBC cells to TRAIL-induced caspase activation and cell death, underscoring the importance of this molecular event in sensitizing TNBC cells to TRAIL. Our results are also consistent with a prior report demonstrating that a small molecule inhibitor of XIAP (embelin) sensitizes inflammatory breast cancer cells lines to TRAIL [37].

Notably, we did not observe any significant effects of metformin on the cell surface expression of TRAIL receptors in contrast to other reports that metformin increases TRAIL-R2 protein levels, although the cellular localization of the receptor was not delineated in these prior studies [29, 31]. Additionally, metformin did not affect protein levels of MAGED2, indicating that

metformin sensitizes TNBC cells to TRAIL receptor agonists by a different mechanism than methionine restriction, which increases cell surface expression of TRAIL-R2 by downregulating expression of MAGED2, an inhibitor of TRAIL receptor expression [14, 38]. Furthermore, other groups have implicated downregulation of the antiapoptotic protein FLIP as an important molecular event in the TRAIL-sensitizing effects of metformin [12, 39]. Although our results do not exclude the potential contribution of FLIP or other molecular targets, our studies point to the downregulation of XIAP by metformin as a functionally relevant event for its TRAIL-sensitizing effects.

In conclusion, we have demonstrated that metformin downregulates XIAP and sensitizes clinically aggressive TNBC cells to TRAIL receptor agonists. We have also shown that the combination of metformin and TRAIL has robust antitumor activity in a murine model of metastatic TNBC which recapitulates features of the human disease. Given the lack of targeted therapies for TNBC and the largely disappointing activity of TRAIL receptor agonists in clinical trials to date, our results point to the combination of metformin and TRAIL receptor agonists as a promising approach to enhance activity of these proapoptotic agents. Finally, our studies suggest that inhibition of XIAP expression by metformin may be a useful biomarker to predict response to this combined therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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